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Postprandial Metabolic and Appetite Responses Following Whey Protein Supplementation at Breakfast

D M Allerton

PhD

2016

Postprandial Metabolic and Appetite Responses Following Whey Protein Supplementation at Breakfast

Dean M. Allerton

A thesis submitted in partial fulfilment of the
requirements of the University of
Northumbria at Newcastle for the degree of
Doctor of Philosophy

Research undertaken in the Faculty of Health
and Life Sciences, Northumbria University

November 2016

ABSTRACT

The prevailing lifestyle pattern in western society results in humans spending the majority of non-sleeping hours in the postprandial state. Given that impaired postprandial metabolism may have adverse consequences for health, interventions that positively influence the postprandial milieu are pertinent considering the global rise in prevalence of chronic metabolic disorders. The aim of this thesis was to investigate whether consumption of whey protein, which has reported beneficial properties relevant to metabolic health, could impact favourably upon acute and second-meal postprandial metabolic and appetite responses. More specifically, a series of studies was designed to establish the conditions under which a rationale for whey protein supplementation may be strongest, through investigation of co-ingestion with other macronutrients, timing of supplementation and interactions with low-moderate intensity exercise.

Study one revealed that consuming whey (20 g) alongside a high-carbohydrate or high-fat breakfast significantly augmented the plasma insulin response to that meal in physically active, normal-weight males, without significantly affecting postprandial glycaemia, lipaemia or subjective appetite. Whey protein consumption at breakfast did not affect second meal responses or 24-hour glycaemia when co-ingested with either macronutrient. Study two compared the effects of the same dose of protein administered before, during or after a mixed-macronutrient breakfast in centrally-obese males. Consuming whey as a preload 15 minutes prior to breakfast significantly reduced postprandial glycaemia compared to consuming it afterwards or not at all, with evidence indicating that insulin-independent mechanisms may

be responsible. Again, prior whey consumption, irrespective of timing, did not influence glycaemic, insulinaemic or appetite profiles following a standard lunch meal. The final study investigated the effects of a post-exercise whey protein preload on postprandial metabolism and appetite in physically inactive, centrally-obese males. Postprandial glycaemia was moderately impaired following brisk walking exercise without supplemental protein, however this effect was negated when exercise was followed by a whey preload, with a reduced peak glycaemic excursion observed compared to other conditions. Ad libitum energy intake, assessed at lunch, did not differ between conditions.

This thesis has shown that whey protein supplementation at breakfast may acutely influence postprandial glycaemia in centrally-obese individuals, however this beneficial effect is not carried over to subsequent meal occasions. Timing of protein intake appears to be important, and this effect is not diminished by prior walking exercise in previously sedentary individuals. Further studies to investigate the effects of whey protein supplementation at multiple meals are required to determine whether this may be a worthwhile strategy to prevent day-long elevated glycaemic exposure.

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LIST OF ABBREVIATIONS

ANOVA: Analysis of variance

ApoB48: Apolipoprotein B48

AUC: area under the curve

BCAAs: Branched-chain amino acids

BMI: Body mass index

CAS: Combined appetite score

CCK: Cholecystokinin

CGM: Continuous glucose monitor

CNS: Central nervous system

CV%: Coefficient of variation

CVD: Cardiovascular disease

DPP-IV: Dipeptyl peptidase-IV

EAA: Essential amino acid

GI: Glycaemic index

GLP-1: Glucagon-like peptide-1

GLUTn: Glucose transporter type n

HbA_{1c}: Glycated haemoglobin

HRP: Horseradish peroxidase

HSL: Hormone-sensitive lipase

IFG: Impaired fasting glucose

IGT: Impaired glucose tolerance

ISAK: International Society for the Advance ment of Kinanthropometry

LPL: Lipoprotein lipase

NEFA: Non-esterified fatty acids

NGT: Normal glucose tolerance

OGTT: Oral glucose tolerance test

PYY: Peptide tyrosine-tyrosine

RPE: Rating of perceived exertion

SD: Standard deviation

SEM: Standard error of the mean

SGLT1: Sodium-glucose linked transporter type 1

SPSS: Statistical Package for the Social Sciences

T2DM: Type 2 diabetes mellitus

VAS: Visual analogue scales

WHO: World Health Organisation

PREFACE

Peer-reviewed publications arising from this thesis:

Allerton, D. M., Campbell, M. D., Gonzalez, J. T., Rumbold, P. L. S., West, D. J., & Stevenson, E. J. (2016). Co-Ingestion of Whey Protein with a Carbohydrate-Rich Breakfast Does Not Affect Glycemia, Insulinemia or Subjective Appetite Following a Subsequent Meal in Healthy Males. *Nutrients*, 8 (3), 116.

Peer reviewed publications during doctoral studies:

Campbell, M. D., Walker, M., King, D., Gonzalez, J. T., **Allerton, D. M.**, Stevenson, E. J., . . . West, D. J. (2016). Carbohydrate counting at meal time followed by a small secondary postprandial bolus injection at 3 hours prevents late hyperglycemia, without hypoglycemia, after a high-carbohydrate, high-fat meal in type 1 diabetes. *Diabetes Care*, 39 (9), e141-e142.

Clifford, T., **Allerton, D. M.**, Brown, M. A., Harper, L., Horsburgh, S., Keane, K. M., . . . Howatson, G. (2016). Minimal muscle damage after a marathon and no influence of beetroot juice on inflammation and recovery. *Applied Physiology, Nutrition, and Metabolism*, 42, 263-270.

Gill, S. K., Hankey, J., Wright, A., Marczak, S., Hemming, K., **Allerton, D. M.**, . . . Costa, R. J. S. (2015). The impact of a 24-h ultra-marathon on circulatory endotoxin and cytokine profile. *International Journal of Sports Medicine*, 36 (8), 688-695.

Gill, S. K., **Allerton, D. M.**, Robson-Ansley, P. J., Hemmings, K., Cox, M., & Costa, R. J. (2016). Does short-term high dose probiotic supplementation containing lactobacillus casei attenuate exertional-heat stress induced endotoxaemia and cytokinaemia? *International Journal of Sport Nutrition and Exercise Metabolism*, 26 (3), 268-275.

Conference proceedings arising from this thesis:

- Allerton, D. M.**, Campbell, M. D., Gonzalez, J. T., West, D. J., & Stevenson, E. J. (2015). The influence of a carbohydrate and whey protein based breakfast on metabolic and appetite parameters following a second meal. *Proceedings of the Nutrition Society*, 74 (OCE3), E198.
- Allerton, D. M.**, Campbell, M. D., Gonzalez, J. T., West, D. J., & Stevenson, E. J. (2015). Addition of whey protein to a fat-based breakfast has detrimental effects on satiety following subsequent feeding. *Appetite*, 91, 435.
- Allerton, D. M.**, Campbell, M. D., Gonzalez, J. T., West, D. J., & Stevenson, E. J. (2015). The effect of breakfast macronutrient content or skipping breakfast on 24h glucose profiles. *Diabetic Medicine*, 32 (S1), 55.
- Allerton, D. M.**, Campbell, M. D., West, D. J., & Stevenson, E. J. (2016). Consuming whey protein before or with breakfast improves postprandial glycaemia in obese men. *Diabetic Medicine*, 33 (S1), 68.
- Allerton, D. M.**, Rumbold, P. L. S., West, D. J., & Stevenson, E. J. (2017). Whey protein consumption following fasted walking exercise reduces subsequent post-breakfast glucose excursions in obese males. *Diabetic Medicine*, 34 (S1), 55.

Conference proceedings during doctoral studies:

- Campbell, M. D., Walker, M., King, D., **Allerton, D. M.**, Shaw, J., Luzio, S., . . . West, D. J. (2016). Administering a small rapid-acting insulin bolus 3 h after eating a mixed macronutrient meal improves postprandial glucose control, without hypoglycaemia, in people with type 1 diabetes. *Diabetic Medicine*, 33 (S1), 109.

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AUTHOR DECLARATION

I declare that the work contained in this thesis comprises all my own work, and that due acknowledgement is made to the opinions, ideas and contributions from the work of others.

I also confirm that this work has not been submitted for any other award.

Ethical clearance for the research presented in this thesis was granted by the Faculty of Health and Life Sciences Research Ethics Committee, Northumbria University.

The word count of this thesis is 45,563 words

Name: Dean M. Allerton

Signature:

Date: 02/12/16

CHAPTER 1

Introduction

1.1 Introduction

The impact of impaired metabolic health is increasingly a global concern which is driven largely by increased prevalence of obesity. In a systematic report published in *The Lancet* using data from 1.9 million participants residing in 199 countries, Finucane *et al.* (2011) estimated that there are 502 million adults with obesity globally. Further reports indicate that prevalence is rapidly increasing in developing countries (Misra & Khurana, 2008) (see Figure 1.1), and the effects of an ‘obesity epidemic’, which began in the USA over 40 years ago (Malik *et al.*, 2013), are recognised by the World Health Organisation (WHO) (James, 2008). According to the most recent Health Survey for England (Bridges *et al.*, 2015) 25.6% of the adult population are obese (body mass index (BMI) $> 30 \text{ kg}\cdot\text{m}^{-2}$).

The consistently high prevalence of obesity has far-reaching effects on society, and the health burden is largely driven by the increased risk of developing type 2 diabetes mellitus (T2DM), cardiovascular disease (CVD) and some forms of cancer (Wang *et al.*, 2011). In line with increasing obesity, it follows that T2DM and metabolic syndrome, a clustering of inter-related metabolic risk factors (Kassi *et al.*, 2011), are becoming more prevalent worldwide (Halcox & Misra, 2015; Nolan *et al.*, 2011). Current estimates predict that the global prevalence of T2DM will rise to 334 million patients by 2025 (Gillett *et al.*, 2012), with

accompanying increases in young people (Elder *et al.*, 2015). Moreover, there are approximately 316 million individuals with impaired glucose tolerance (IGT) who are at high risk of progression to T2DM (Verma & Hussain, 2016). The impact of such disorders upon quality of life, in addition to the health care burden (treatment and management of diabetes and its ensuing complications accounts for approximately 10% of National Health Service spending per annum (Diabetes UK, 2014; Hex *et al.*, 2012)), underscores the importance of implementing strategies to avert the progression from normal to impaired metabolism.

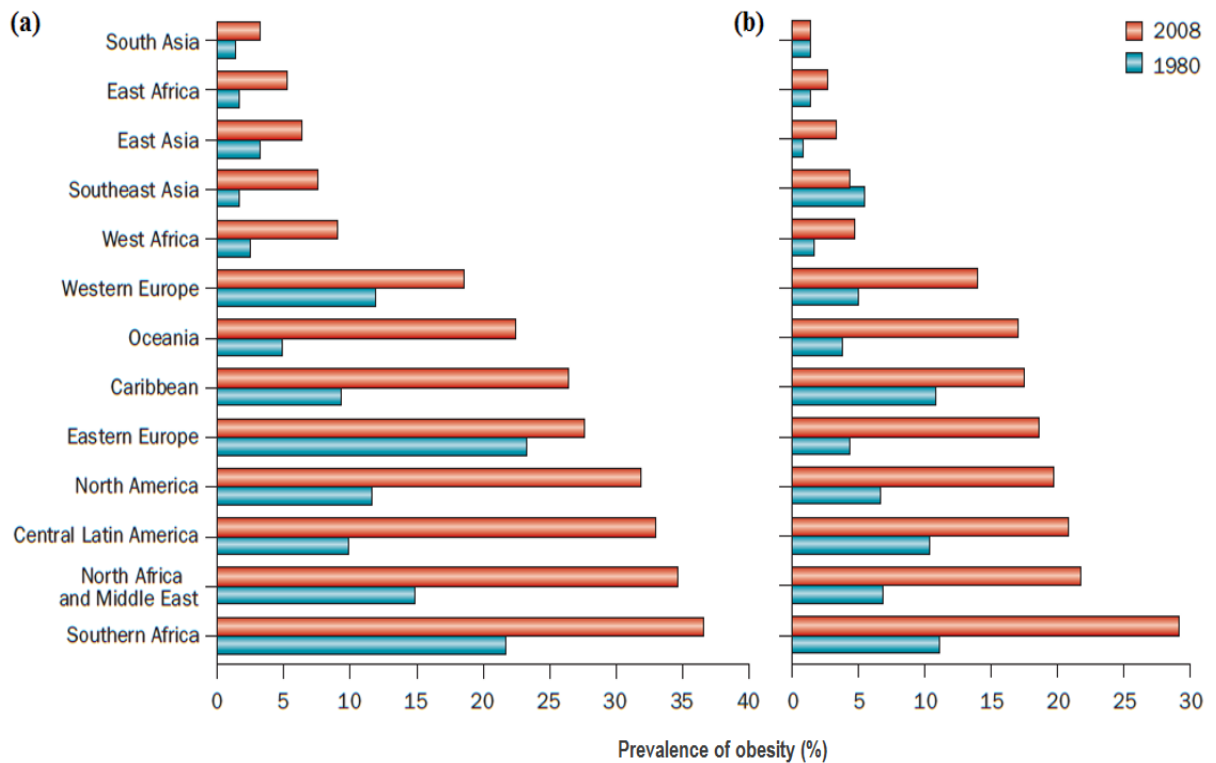


Figure 1.1 The prevalence of obesity in adult women (a) and men (b) in 1980 and 2008 from different world regions. Adapted from Finucane *et al.* (2011) and Malik *et al.* (2013).

Pharmacological agents such as metformin and thiazolidinediones can be effective in lowering blood glucose and reducing the risk of metabolic diseases, with possible favourable cardiovascular effects, however their efficacy over the long term is uncertain and side effects limit their use (Tahrani *et al.*, 2011; Tahrani *et al.*, 2010). The role of lifestyle change in the prevention of onset and deterioration in metabolic health is therefore paramount, and remains the cornerstone in prevention and management of insulin resistance (Altaf *et al.*, 2015). Evidence demonstrating that many chronic diseases are preventable through lifestyle is compelling (Diabetes Prevention Program Research Group, 2002; Salas-Salvadó *et al.*, 2011; Tuomilehto *et al.*, 2001), thus health promotion strategies are necessary in improving quality of life and reducing the burden on health care systems (Slawson *et al.*, 2013). A key part of this is identifying dietary practices as well as foods or food components which facilitate reduced risk of metabolic health deterioration.

Epidemiological studies have demonstrated an inverse relationship between dairy product consumption and incidence of obesity (Mirmiran *et al.*, 2005; Rautiainen *et al.*, 2016), metabolic syndrome (Drehmer *et al.*, 2015a) and T2DM (Aune *et al.*, 2013; Díaz-López *et al.*, 2016). It has been proposed that dairy proteins, particularly whey protein, may be responsible for driving this association. Whey protein comprises approximately 20% of bovine milk protein and was historically seen as a waste by-product of the cheese manufacturing process, where the solid curds (casein protein constituting the remaining 80%) are removed from milk leaving the soluble whey proteins behind (Bansal & Bhandari, 2016). It contains a heterogeneous group of protein fractions, a number of which have been shown to exert biological effects *in vitro* (Lacroix & Li-Chan, 2014) and in animal models (Shi *et al.*, 2012). Whey is now recognised as a high-quality protein and a valuable co-product of

casein production for use as a functional food ingredient (Luhovyy *et al.*, 2007), with an increasing number of experimental studies investigating the effects of whey consumption on metabolic regulation, satiety and food intake. However, many questions remain regarding the effectiveness of whey protein to augment meal responses, and knowledge of beneficial strategies for its supplementation which have ‘real world’ application may be important when evaluating the efficacy of whey protein to help prevent the progression of metabolic impairment.

CHAPTER 2

Literature Review

2.1 Introduction

Given the vast global burden of poor metabolic health on both society and healthcare systems, it is prudent to consider lifestyle and dietary interventions to prevent or manage the deterioration of metabolic processes associated with the onset of obesity and related metabolic disorders. The following review of literature therefore presents an overview of normal metabolic and appetite regulatory processes, how they are impaired in declining metabolic health, and strategies to prevent or manage this decline.

2.2 Normal metabolism

2.2.1 Carbohydrate metabolism

Carbohydrate metabolism involves coordinated regulation of glucose intake, storage, mobilisation and breakdown (Efeyan *et al.*, 2015). Synchronised adjustments are made to rates of glucose appearance (from exogenous and endogenous pathways) into and disappearance (tissue uptake and storage) from the blood, to manipulate glucose flux and thus maintain blood glucose concentration within the typical range.

In the postabsorptive state, typically represented by an overnight fast, glucose turnover decreases to its nadir within the 24-hour cycle, and plasma glucose concentration is relatively stable (Shrayyef & Gerich, 2010). The greatest test of glucoregulation occurs when this situation is challenged by ingestion of a meal (particularly a high carbohydrate meal), and gives way to the postprandial state. This period of 4-7 hours immediately following meal ingestion involves nutrient assimilation and metabolism (Gerich, 1993; Monnier, 2000). Dietary carbohydrates are digested via progressive hydrolysis in the intestinal lumen and a number of enzymes located within the brush-border of enterocytes, in order to liberate free monosaccharides (Monnier, 2000). These are conveyed into the enterocyte via transport proteins, and ultimately enter the circulation into the hepatic portal vein.

Blood glucose concentration is noticeably increased within 15 minutes of ingestion of a mixed meal, and peaks at around 30-60 minutes (Frayn, 2010). The size of postprandial glycaemic excursions from postabsorptive levels has become a prominent area of interest for researchers, as postprandial hyperglycaemia is considered a precursor for T2DM and an independent risk factor for CVD (Gerich, 2006). The extent of circulating glucose excursions following meal ingestion may depend on a number of factors. Firstly, the rate of gastric emptying is known to account for up to 35-50% of the variation in peak blood glucose following ingestion of glucose in healthy individuals (Horowitz *et al.*, 1993; Woerle *et al.*, 2008), and can be altered by aspects such as meal size and the amounts of complex carbohydrate, fibre and simple sugars in the meal (Russell *et al.*, 2016). Other factors include whether the meal is in solid or liquid form, and the accompanying amounts of protein and fat, both of which delay gastric emptying (Gerich, 1993).

Insulin is a key metabolic regulator, acting primarily to lower plasma glucose concentration via suppression of endogenous glucose production from the liver and kidneys, promotion of glucose uptake into muscle and adipose tissue and inhibition of release of non-esterified fatty acids (NEFA) into the circulation. Insulin synthesis and secretion occurs within pancreatic β -cells and glucose concentration is the main regulator of its secretion (Stumvoll, 2004), with a three- to fourfold increase in plasma insulin from basal level observable within 30-60 minutes of meal ingestion (Shrayyef & Gerich, 2010). Glucose enters β -cells via glucose transport proteins (GLUT1-3) at a rate determined by its concentration (Yang, 2014).

Insulin acts to restrain hepatic glucose output indirectly by inhibiting glycogen phosphorylase while simultaneously promoting glycogen synthase, thus a switch in emphasis from glycogen breakdown to glycogen storage occurs (Frayn, 2010). This is a relatively slow process, and in order to promote a reduction in plasma glucose, uptake into other tissues is required. Muscle and adipose glucose uptake is dependent upon the transport of glucose into cells, which is mediated by insulin-sensitive glucose transporter type 4 (GLUT4). An increase in circulating insulin following a meal leads to binding of insulin to its receptor on the cell surface, generating a complex signalling-cascade which induces rapid translocation of GLUT4 to via membrane fusion (Yang, 2014).

As time since meal ingestion proceeds, and an individual begins to advance back into the postabsorptive state, plasma insulin levels decline and glucagon levels rise. Counter-regulation of glucose is assisted by increased release of the catecholamines adrenaline and noradrenaline during stress or hypoglycaemia, with cortisol and growth hormone becoming important with more severe or prolonged hypoglycaemia (Mitrakou *et al.*, 1991).

2.2.2 Lipid metabolism

The majority of fat consumed in the diet is in the form of triglyceride. Digestion and absorption of dietary fat results in monoglycerides and fatty acids being taken up into enterocytes where re-esterification occurs, forming new triglyceride molecules. (Hussain, 2014). These are packaged with phospholipids, cholesterol and apolipoprotein-B48 (ApoB48) to produce large lipoprotein molecules (Ansar *et al.*, 2011). In contrast to absorbed carbohydrate, these triglyceride-rich chylomicrons initially bypass splanchnic tissues by exiting absorptive cells via exocytosis into the lacteals, ultimately entering the general circulation at the thoracic duct (Hussain, 2014). This chylomicron pathway is considerably slower than glucose absorption, with peak plasma triglyceride occurring 3-4 hours following a meal containing a moderate amount of fat (Silva *et al.*, 2005), however short and medium-chain fatty acids (10 carbons or fewer (Bonham *et al.*, 2013)) escape re-esterification and enter the portal circulation directly in the form of NEFA (Bugaut, 1987).

Following an overnight fast plasma NEFA levels are usually at their highest in the 24-hour cycle (around $0.4\text{--}1.0\text{ mmol}\cdot\text{l}^{-1}$ (Kruszynska, 1997)). Despite the lower basal concentration in comparison to glucose, NEFA is the predominant fuel in the postabsorptive state when considering the relative rate of turnover and energy yield of each substrate (Frayn, 2010). NEFA enters the plasma predominantly from the hydrolysis of triglyceride within the adipocyte (Karpe *et al.*, 2011) which occurs as part of a constant cycle of lipolysis and re-esterification. The hydrolysis of triglyceride is carried out by a series of lipases, including hormone-sensitive lipase (HSL) which is potently inhibited by insulin (Saponaro *et al.*, 2015). Consumption of a typical breakfast meal almost maximally suppresses lipolysis

(Chowdhury *et al.*, 2015). In concert with inhibition of HSL activity, the milieu of increasing insulin and glucose concentrations seen postprandially also stimulates adipose tissue glucose uptake and oxidation, with a resultant production of glycerol 3-phosphate (Saponaro *et al.*, 2015). When this is readily available the rate of re-esterification of NEFA will be high, thus the rate of release of NEFA into the circulation is reduced (Goodman, 2009), and there is a subsequent switch to the use of glucose as the predominant fuel (Frayn, 2010).

Insulin also has a role in promoting fat storage following consumption of a mixed meal. The majority of triglyceride is removed from the circulation by adipose tissue, facilitated by lipoprotein lipase (LPL) which adheres to the epithelium of its associated capillaries (Goodman, 2009). Insulin stimulates adipose LPL, mediated via amplified transcription and increased export to endothelial cells, indicating that this is not an immediate effect. Consequently, LPL activity is greatest after several hours of insulin stimulation (Czech *et al.*, 2013) which coincides with chylomicron entry into the plasma, allowing rapid clearance and emphasising the coordination of insulin secretion with lipid metabolism (Frayn, 2010).

2.2.3 Protein metabolism

Proteins are fundamental to both the architecture and the activity of living cells, functioning as structural and contractile elements as well as hormones, enzymes, transport proteins, antibodies and numerous other physiological roles (Gropper & Smith, 2013). In the postabsorptive state, there is net breakdown of protein in muscle, which can largely be attributed to the low insulin concentration. Insulin is perhaps the most important regulator of protein metabolism and has a net anabolic effect, promoting protein synthesis in the setting

of adequate amino acid availability (Biolo *et al.*, 1999). Following a meal containing protein, plasma amino acids enter the circulation within 15-30 minutes and the concentration typically rises for several hours before returning to basal levels (Frayn, 2010). Up to a third of dietary amino acids are metabolised by splanchnic tissues (Soultoukis & Partridge, 2016), however branched-chain amino acids (BCAAs) typically remain in the circulation. Indeed, despite making up ~20% of dietary protein intake, BCAAs account for ~70% of amino acids leaving the liver following a meal (Kruszynska, 1997).

The utilisation of amino acids is largely influenced by hormonal control. Following a mixed meal containing glucose, it is clear that plasma insulin will rise to affect this change in protein metabolism, however amino acids are also able to stimulate insulin secretion from β -cells (Newsholme *et al.*, 2005), although the various mechanisms for this effect remain to be fully elucidated. This effect is noteworthy when considering individuals with T2DM, who have a blunted insulin response to increases in plasma glucose concentration, but amplified insulin secretion following amino acid ingestion (van Loon *et al.*, 2003). This insulinitrophic effect appears greater following protein and carbohydrate coingestion rather than consumption of either macronutrient alone (Gropper & Smith, 2013).

2.2.4 Acute effects of aerobic exercise on subsequent metabolism

Regular aerobic exercise is considered an effective strategy in the prevention of metabolic health impairment, partly due to the transient effects on postprandial metabolism which can last upwards of 48 hours following the most recent bout (Colberg *et al.*, 2010). Acute exercise is known to significantly impact lipid metabolism. Whilst triglyceride levels may remain

unchanged or slightly increase during exercise in the fasting state (Maraki & Sidossis, 2013), studies have consistently reported a reduction in triglyceride response following a high-fat meal after prior exercise (Freese *et al.*, 2014). The effect of aerobic exercise on postprandial lipaemia appears to be related to the energy deficit created by exercise (Gill *et al.*, 2002), independent of the duration or intensity of exercise, and the effects are attenuated when expended energy is replaced following exercise (Burton *et al.*, 2008). The mechanisms responsible remain uncertain, however it is likely that increased clearance of triglyceride-rich lipoprotein particles via enhanced muscle LPL activity plays a part, driven by the need to replenish intramuscular triglyceride stores depleted by exercise (Maraki & Sidossis, 2013). When individuals who have undergone a 12 week brisk walking programme were challenged to a high-fat meal, the postprandial lipaemic response did not differ from controls when administered 48 hours after exercise (Aldred *et al.*, 1995), indicating that the effects of exercise on triglyceride metabolism appear to be transient and quickly reversed.

It is well established that a single bout of aerobic exercise is sufficient to increase insulin sensitivity (Horowitz, 2007; Nelson & Horowitz, 2014), however the specific effects on subsequent postprandial glycaemic control are less well known. Whilst it would be expected that an increase in post exercise muscle glucose uptake would lead to improvements in post-exercise glycaemia, responses to a mixed meal or oral glucose tolerance test (OGTT), assessed close to cessation of exercise, have included improvements (Hasson *et al.*, 2010), no change (Gonzalez *et al.*, 2013) or decrements (Rose *et al.*, 2001) in glucose tolerance. Differences are likely to be affected by the fitness and prior nutritional state of the participant, exercise modality, and the timing and method of glucose tolerance assessment. An increase in whole body glucose uptake, indicated by a 24% greater increase in rate of glucose

disappearance in an OGTT following prior exercise (Rose *et al.*, 2001), is likely due to the drive for muscle glycogen repletion, mediated by the residual effects of contraction-induced GLUT4 translocation (Borghouts & Keizer, 2000). Enhancement in the rate of glucose appearance from meal-derived carbohydrate appears to also be elevated in the postprandial period following prior exercise, and may supersede the rate of disappearance leading to greater rises plasma glucose (Rose *et al.*, 2001), with greater post-exercise intestinal absorption a likely mediator (Gonzalez, 2014). Knudsen *et al.* (2014) recently established that the effect of an acute bout of moderate-intensity exercise appears to be influenced by underlying glycaemic control, with an immediate increase in the postprandial glucose response observed in normoglycaemic participants, contrasting with preservation of glucose tolerance in those with IGT and T2DM. The mechanisms underlying such an effect remain unclear however, and the effect of altering post-exercise nutritional intake is uncertain.

Ultimately, the relative significance of the acute effects of exercise on subsequent glycaemia may depend on whether an acute bout of exercise is followed up by further repeated exercise. Regular exercise training is commonly accompanied by alterations in aerobic fitness and body mass which have an independent influence on glucose metabolism (Coker *et al.*, 2009). In sedentary non-diabetic individuals who are unlikely to undertake frequent bouts of exercise or physical activity, adjusting post-exercise macronutrient intake may be a prudent strategy to modulate post-exercise glycaemia.

2.3 Appetite and energy intake

Control of appetite involves the complex processing of a series of homeostatic and hedonic stimuli regulating the initiation and maintenance of food ingestion (Berthoud, 2006). In addition to internal signals generated by physiological processes, external cues related to food and the food consumption environment exert effects on subjective perceptions of hunger and satiety through psychological processes (Mela, 2006). Total daily energy intake is a function of both the number of eating events and meal size (Benelam, 2009). Satiation, defined as the process leading to termination of eating, and satiety, defined as the process leading to inhibition of further eating (Benelam, 2009; Blundell *et al.*, 2010), therefore play important roles in influencing the amount consumed at, and the time period between, each meal occasion.

The numerous stimuli modulating satiation and satiety are presented in Figure 2.1. Here, satiation and early satiety are influenced largely by several sensory and cognitive factors relating to prior associations and aspects of meal quality. As meal contents progress into the stomach, increased gastric volume induces distension of the stomach which is communicated to the brain via activation of vagal afferents (Badman & Flier, 2005; Wang *et al.*, 2008), enhancing satiation, while the presence of nutrients in the intestinal lumen stimulates secretion of satiety-promoting gastrointestinal hormones (Tremblay & Bellisle, 2015) (discussed further in section 2.3.1). A plethora of intracellular and extracellular nutrient-sensing mechanisms exist throughout the body to detect changing metabolite levels (Efeyan *et al.*, 2015) and following absorption these provide information about nutrient status which may also affect satiety (Benelam, 2009). The influence of blood glucose on appetite has long

been debated since the development of the ‘glucostatic theory’ by Mayer (1953), however evidence for a role of circulating glucose on appetite regulation under normal physiologic conditions is equivocal (Flint *et al.*, 2007; Schultes *et al.*, 2016). Longer term signals, including insulin and the adipokine leptin provide information regarding energy status (see section 2.3.2) in order to influence energy balance (Woods & D'Alessio, 2008).

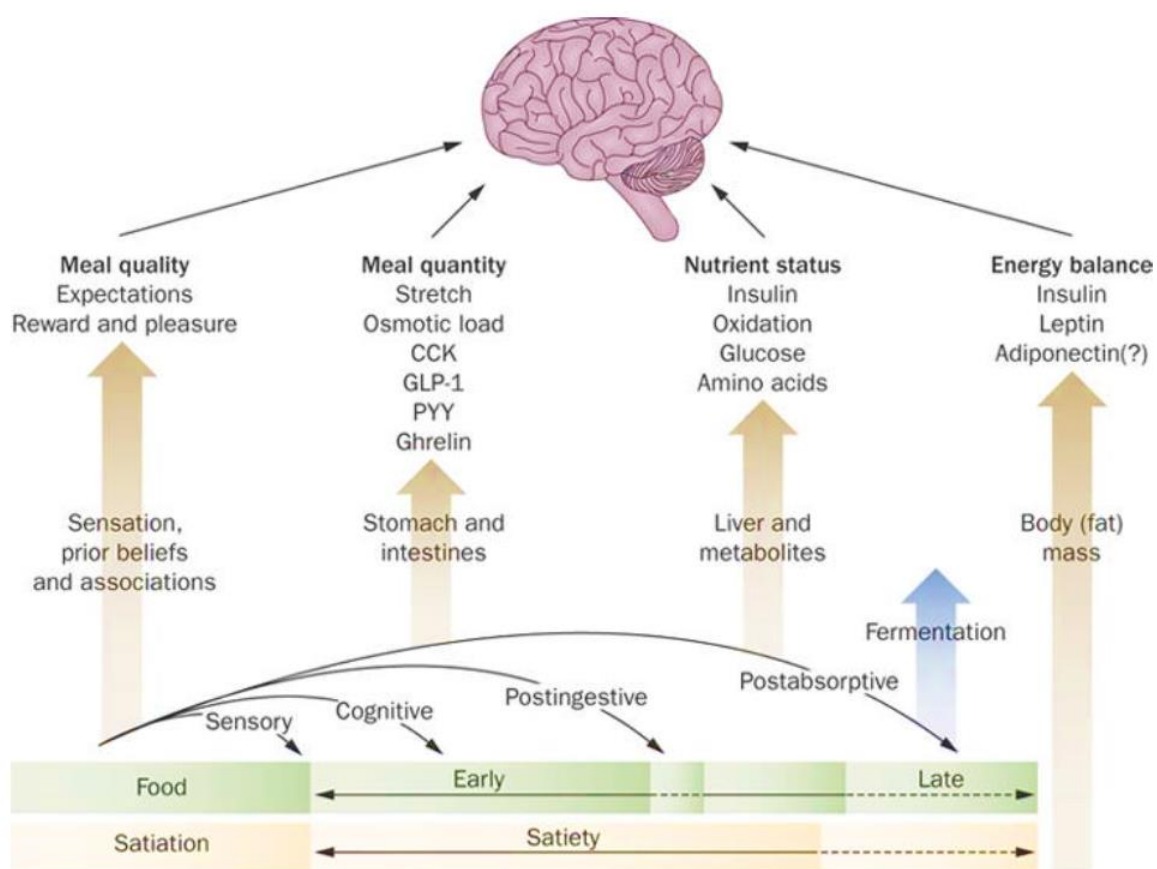


Figure 2.1 The 'Satiety Cascade' illustrating the cognitive and physiological processes influencing satiation and satiety. CCK, cholecystokinin; GLP-1, glucagon-like peptide; PYY, peptide tyrosine-tyrosine. Adapted from Blundell *et al.* (2010).

Whilst the predominant focus of this thesis is on postprandial metabolic responses, modulation of appetite responses may clearly be of importance in the context of metabolic

health and subsequent energy intake. Physiologic changes in homeostatic appetite signals were not measured as part of this work, however considering their importance in the regulation of appetite, it is pertinent to briefly discuss their various functions in post-ingestive control of energy intake.

2.3.1 Episodic appetite hormones

A number of hormones act in the short term as episodic satiation or satiety signals, so-called because they act in unison with episodes of eating (Stensel, 2010), and these are summarised in Table 2.1. Of these, ghrelin is the only orexigenic (hunger stimulating) hormone currently identified and its suppression is relevant to the onset of satiety (Benelam, 2009). Levels of circulating ghrelin rise during fasting and immediately prior to meals but fall rapidly following meal intake (Cummings *et al.*, 2004; Cummings *et al.*, 2001), with suppression proportional to the caloric load ingested (Callahan *et al.*, 2004).

In contrast to the orexigenic effects of ghrelin, several gastrointestinally-derived peptides are known to have anorexigenic (appetite suppressing) effects. Glucagon-like peptide-1 (GLP-1) is secreted from the L-cells of the intestinal mucosa following direct contact with nutrients, in proportion to the caloric load and glucose in particular (Ahrén *et al.*, 2010). A meta-analysis of nine datasets (Verdich *et al.*, 2001) identified a dose-dependent link between GLP-1 infusion, reductions in food intake and enhanced satiety in lean and obese participants, although obese individuals may be less responsive to its actions (Holst, 2007). The presence of nutrients in the duodenum and jejunum initiates rapid release of cholecystokinin (CCK) into circulation, particularly in response to fat- or protein rich meals which acts to suppress

intake during meals (Woods & D'Alessio, 2008). Peptide tyrosine-tyrosine (PYY) is co-secreted from L cells in the distal gut alongside GLP-1 (Suzuki *et al.*, 2012) and inhibits food intake, shown by a dose-dependent reduction in subsequent meal size following exogenous infusion (Muurahainen *et al.*, 1988). An attenuated meal-stimulated PYY response is observed in obesity, with higher energy intake required to achieve similar postprandial PYY concentrations (Batterham *et al.*, 2006).

Table 2.1 Summary of relevant episodic appetite hormones and their actions. Adapted from Benelam (2009) and Suzuki *et al.* (2010).

Hormone	Primary sites of secretion	Effect on appetite	Mechanism	Additional effects
Ghrelin	Stomach	↑ Hunger	Via ghrelin receptors in the brain	Long-term effect on energy balance Growth hormone secretion
CCK	Duodenum and jejunum	↑ Satiation	Via vagus nerve	Delays gastric emptying Stimulates pancreatic enzyme secretion Stimulates gall bladder contraction Acts as a neurotransmitter
GLP-1	L-cells of intestine and brain	↑ Satiety	Via GLP-1R in brain	Incretin (stimulates insulin production) Slows gastric emptying
PYY ₃₋₃₆	Ileum, colon and rectum	↑ Satiety	Via Y2 receptors in brain	Slows gastric emptying Delays intestinal transport Reduces gastric secretions
Glucagon	Pancreatic α -cells	↑ Satiety	Via vagus nerve	Increases blood glucose levels and insulin secretion

CCK, cholecystokinin; GLP-1, glucagon-like peptide-1; PYY, peptide tyrosine-tyrosine; GLP-1R, glucagon-like peptide-1 receptor.

2.3.2 Tonic appetite hormones

Longer term regulation of appetite is influenced by tonic signals of adiposity which act at the arcuate nuclei in the hypothalamus. These signals provide a background tone that determines

the sensitivity of the brain to satiation signals, thereby subtly influencing energy intake at a given meal (Woods & D'Alessio, 2008). The discovery of Leptin by Zhang *et al.* (1994) was instrumental in developing understanding of adipose tissue as an endocrine organ. It is secreted by white adipose tissue, with circulating levels that are proportional to fat mass in weight-stable individuals (Considine *et al.*, 1996), but are reduced by weight loss (Hussain & Bloom, 2013). Leptin inhibits orexigenic neurons and activates anorexigenic neurons (Suzuki *et al.*, 2012). These express leptin receptors, and subsequent interactions have the net effect of reducing food intake and increasing energy expenditure (Park & Ahima, 2015). Leptin deficiency is associated with severe obesity, however obese individuals commonly present with high levels of leptin, leading to the concept of leptin resistance (Neary *et al.*, 2004). Thus treatment of individuals with exogenous leptin is of limited benefit to those who do not suffer from congenital lipid deficiency (Farr *et al.*, 2015; Mantzoros & Flier, 2000).

Insulin is a key regulator of energy homeostasis and primarily acts to lower elevated plasma glucose as described in section 2.2.1, however it also acts as a tonic signal of energy status in the central nervous system (CNS). Both fasting and postprandial levels of insulin are proportional to the fat mass of the individual (Neary *et al.*, 2004). Insulin receptors populate similar neuronal populations within the brain as leptin (Arora & Anubhuti, 2006), hence these neurons receive feedback indicating the adequacy of energy stores within the body from both hormones (Blundell *et al.*, 2015). Insulin resistance is characteristic of the obese state (Woods & D'Alessio, 2008), thus the strength of tonic inhibition of fat mass on food intake is weakened (Blundell *et al.*, 2015), indicated by the association between insulin and appetite reported in healthy volunteers being blunted in overweight individuals (Flint *et al.*, 2007).

2.3.3 Measurement of appetite

The physiological regulators of appetite provide a variety of internal signals which are interpreted as subjective feelings associated with hunger and satiety (Bilman *et al.*, 2015; Stubbs *et al.*, 2000). These feelings are commonly measured using visual analogue scales (VAS) to quantify different aspects of appetite. These consist of unipolar unstructured line scales anchored with descriptive statements of opposing meaning (Blundell *et al.*, 2010) to capture sensations of appetite-related aspects such as hunger, fullness, satiety and prospective food consumption. This method is easy to administer and imposes little burden upon the participant, however several theoretical issues are associated with their use. By definition, appetite sensations are subjective constructs making comparison between participants difficult to interpret (Livingstone *et al.*, 2000), however use of within-subject designs can overcome such inter-individual differences. In free-living conditions people do not just eat when ‘metabolically hungry’, indeed it is common for individuals to initiate feeding in the absence of hunger and in spite of large fat reserves (Berthoud, 2011), often linked to cognitive and emotional factors, and VAS should therefore not be considered as a proxy for measures of energy intake. Whilst the association with food intake has been described as modest (Benelam, 2009), variation in subjective appetite measures has previously been associated with subsequent intake (Drapeau *et al.*, 2007; Flint *et al.*, 2000; Parker *et al.*, 2004).

To provide further insight into the impact of an intervention on appetite and feeding behaviour, measurements of food intake are often taken alongside subjective appetite responses (Stubbs *et al.*, 2000). Laboratory assessments of *ad libitum* energy intake at a single meal can be quantified using a free choice buffet or a fixed meal of known macronutrient

composition. Too much choice may undermine physiological satiety signals (Livingstone *et al.*, 2000), thus current recommendations advise the avoidance of buffet style test meals unless there is a specific hypothesis relating to food choice (Blundell *et al.*, 2010). The laboratory setting enhances the efficacy of both methods by eradicating extraneous influences on satiety that are encountered in the free-living setting (Benelam, 2009). Alternatively, the use of self-reported weighed or estimated food diaries theoretically increases external validity by assessing intake under normal conditions (Blundell *et al.*, 2010), however the associated lack of control over the environment in which food is consumed can make interpretation of data problematic. This method is exposed to bias and misreporting, and underestimation of energy intake is common (Livingstone *et al.*, 2000; Livingstone & Black, 2003). A 12% underrecording bias has been observed in obese men when comparing estimated food intake against the doubly labelled water technique (Goris *et al.*, 2000). The use of self-reported food diaries is therefore often reserved for monitoring of pre-trial dietary standardisation or for longer term (24 hour or 7 day) periods of dietary assessment.

2.4 Impaired metabolism

2.4.1 Metabolic disease states

Regulation of blood glucose concentration within a relatively narrow range is a fundamental physiological process, however its derangement over time may lead to progressive states of metabolic impairment and ultimately manifest as T2DM (Stumvoll, 2004). This complex metabolic and endocrine disorder is characterised by hyperglycaemia and altered lipid

metabolism (Nolan *et al.*, 2011) and, if poorly controlled, can lead to a number of complications. These include microvascular disease encompassing nephropathy, neuropathy and retinopathy (Altaf *et al.*, 2015) in addition to macrovascular disorders such as CVD, stroke or peripheral vascular disease (Gillett *et al.*, 2012). The diagnosis of T2DM may be made based on plasma glucose concentration in addition to glycated haemoglobin (HbA_{1c}) and clinical judgement of risk factors such as family history. Plasma glucose during fasting or following an OGTT may be compared to standardised cut-off values (Table 2.2) to assess whether glycaemia falls within normal, impaired or diabetic regions (American Diabetes Association, 2016).

Table 2.2 Criteria for the diagnosis of diabetes and categories of increased risk. Data adapted from American Diabetes Association (2016).

Category	Plasma glucose concentration (mmol·l ⁻¹)*	
	Fasting	2 hour
Normoglycaemic	< 5.6	< 7.8
Impaired fasting glucose	5.6 - 6.9	-
Impaired glucose tolerance	-	7.8 - 11.0
Diabetes mellitus	≥ 7.0	≥ 11.1

*Fasting, no caloric intake for at least 8 hours; 2 hour, following 75 g oral glucose tolerance test.

The long-established belief that T2DM is a progressive condition that can be managed and decelerated, but not reversed, has been challenged in recent years. Studies using hypocaloric diets have demonstrated normalisation of β -cell function and hepatic insulin sensitivity in T2DM of short duration (Lim *et al.*, 2011) and fasting glucose in 50% of participants with T2DM of longer duration (Steven & Taylor, 2015). However, the extreme nature of these

interventions (maintaining an ~2.5 MJ (600 kcal) per day diet regimen for at least 8 weeks) suggests that this may not be a realistic option for all individuals (Taylor, 2013), and serves to emphasise the significance of preventing the decline in metabolic health from normal to impaired, and subsequently to T2DM.

Individuals who present with plasma glucose levels that are higher than normal, but not elevated to diabetic levels, have been termed ‘pre-diabetic’ (American Diabetes Association, 2016). Whether or not this constitutes a disease state in itself is the subject of much debate (Grundy, 2012). Regardless of the terminology, those with impaired fasting glucose (IFG) and/or IGT (Table 2.2) display an increased risk of future development of T2DM and CVD. Such impairments in glycaemic control are indicative of insulin resistance (Grundy, 2012), indeed pre-diabetes is associated with the presence of both insulin resistance and early β -cell dysfunction, abnormalities that often transpire prior to the detection of noticeable changes in blood glucose (Tabák *et al.*, 2012). Insulin resistance is frequently accompanied by visceral obesity, dyslipidaemia and hypertension and this clustering of interrelated CVD risk factors, first postulated by Reaven (1988) as ‘syndrome X’, is now termed ‘metabolic syndrome’.

Several organisations have previously produced diagnostic criteria for metabolic syndrome (Alberti & Zimmet, 1998; Alberti *et al.*, 2005; Grundy *et al.*, 2005; National Cholesterol Education Program, 2002), however some discrepancies in their sensitivity have been recognised (Hunt *et al.*, 2004) and efforts to form a consensus have since resulted in a harmonised set of criteria (Alberti *et al.*, 2009). An additive effect of increasing metabolic syndrome components on risk is beginning to be recognised (O'Neill & O'Driscoll, 2015), such that when metabolic syndrome is combined with pre-diabetes (i.e. the number of

abnormal components is increased), the risk of incident T2DM is even higher (Ford *et al.*, 2008), suggesting the importance of efforts to improve metabolic health before this progression has taken place (Phillips, 2014).

2.4.2 The progression of impaired fuel metabolism

Insulin sensitivity fluctuates throughout the normal life course, and insulin resistance may occur during puberty, pregnancy and as part of ageing (Kahn *et al.*, 2006). However, by far the most prevalent cause, particularly in developed countries, is obesity (Boden, 2011). The presence of insulin resistance, decreased β -cell secretory capacity and concomitant hyperglycaemia in the context of prolonged fat excess has been well established, however the precise mechanisms underpinning the effect of such lipotoxicity on impaired glucose metabolism are the subject of much investigation. The deposition of lipid in insulin target tissues other than adipose tissue, often termed ‘ectopic fat’, may provoke lipid-induced disruption of insulin signalling pathways, and has been proposed as a theory explaining the decrease in insulin action associated with obesity (Shulman, 2014). This is based on the finding that an increase in plasma NEFA leads to intramyocellular and intrahepatic accumulation of triglyceride and NEFA metabolites (Boden *et al.*, 2001), caused by a spillover of energy storage from adipose tissue to other organs following prolonged periods where energy intake exceeds energy expenditure (Shulman, 2014).

Recent evidence also suggests that insulin resistance is related to a state of chronic, low-grade inflammation and macrophage infiltration in adipose tissue, with increased circulating concentrations of pro-inflammatory cytokines associated with obesity (Kwon & Pessin,

2013; Ouchi *et al.*, 2011). In this respect, the distribution of adipose may be important since visceral fat tissue expresses higher levels of many cytokines than subcutaneous depots (Fried *et al.*, 1998). In addition, the rate of lipolysis is higher in visceral adipocytes (Bano, 2013), thus the liver is directly exposed to high levels of both NEFA and pro-inflammatory factors released from visceral fat into the portal vein. The so called ‘portal theory’ dictates that this effect directly leads to elevated hepatic triglyceride and consequent deleterious effects on hepatic insulin sensitivity (Item & Konrad, 2012).

In addition, the overwhelming of storage available in adipocytes leads to spillover of NEFA into plasma which, in concert with cytoplasmic triglyceride, inhibits insulin-stimulated glucose uptake into the muscle and impairs glucose disposal (Miranda *et al.*, 2005). Insulin resistance in adipose tissue also limits postprandial suppression of NEFA mobilisation, with consequent day-long elevation in plasma NEFA (Abdul-Ghani & DeFronzo, 2010; Nolan *et al.*, 2011). The prolonged exposure of β -cells to elevated levels of NEFA and its metabolites (e.g. acetyl coenzyme A) is considered the underlying cause of declining β -cell function (Taylor, 2013). Furthermore, total possible insulin secretion is limited via β -cell apoptosis which is stimulated by chronic exposure to NEFA metabolites (Cnop, 2008). Thus, in susceptible individuals, a vicious cycle is established whereby development of insulin resistance increases the demand for insulin secretion on β -cells however, as β -cell function declines, circulating concentrations of glucose, NEFA and other metabolites increase, exacerbating the cellular damage and further impairing insulin action (Bano, 2013).

2.4.3 The significance of postprandial hyperglycaemia

In the years that precede the development of T2DM, a progressive transition from normal glucose tolerance (NGT) to IGT occurs, characterised by a decline in both insulin action and early phase insulin secretion (Monnier *et al.*, 2007). Hyperglycaemia ensues, with postprandial rather than fasting glucose appearing to deteriorate first (Monnier *et al.*, 2007; Woerle *et al.*, 2004). Evidence suggests a considerable number of individuals with normal fasting glucose will have an abnormal postprandial glucose level (DECODE Study Group, 2001; Gerstein, 2001) and postprandial hyperglycaemia has been identified as an independent and continuous risk factor for CVD in diabetic and non-diabetic populations (Bianchi *et al.*, 2008; Coutinho *et al.*, 1999; Gerich, 2003), even when fasting glucose is in the normal range (Ning *et al.*, 2012). In non-diabetic individuals it appears that increased postprandial glucose is a stronger predictor of HbA_{1c} than increased fasting glucose (Fava, 2008), and also carries a greater risk of CVD (Bianchi *et al.*, 2008; Gerich, 2003).

The mechanisms responsible for the increase in risk of CVD are not fully understood, however endothelial dysfunction has been proposed as a likely facilitator. Glucose enters endothelial cells via insulin-independent GLUT1, making them vulnerable to the deleterious effects of hyperglycaemia in individuals with IGT (Fava, 2008). Large postprandial excursions are thought to be a risk factor even after correcting for mean blood glucose (Colette & Monnier, 2007). Glycaemic exposure and variability, both features of IGT, may contribute towards the development of atherosclerosis through generation of atherogenic particles via glycosylation of proteins in addition to the triggering of endothelial dysfunction and oxidative stress (Bianchi *et al.*, 2008; Ceriello *et al.*, 2008).

IGT is associated with a blunted insulin response to a meal, however it is not necessarily impaired β -cell function that is fully responsible, since a loss of efficiency of the incretin response has been observed previously in individuals with IGT. This long recognised effect describes the phenomenon whereby oral glucose ingestion provokes a greater insulin secretory response than intravenous delivery of the same glucose load (Elrick *et al.*, 1964). In addition, GLP-1 inhibits glucagon secretion and delays gastric emptying (Nadkarni *et al.*, 2014), thereby influencing postprandial metabolism via multiple pathways. Whilst IFG is associated with normal insulin secretion but resistance to insulin action, in IGT both secretion and sensitivity are impaired (Bock *et al.*, 2006), leading to reduced disposal of exogenous glucose with exaggerated and prolonged increases in plasma glucose concentration.

Postprandial hyperglycaemic excursions may be both a self-perpetuating cause, and a function of, the deterioration in fuel homeostasis which increases the risk of CVD and, if left unchecked, commonly leads to development of T2DM. In this context, and considering that progression from NGT to T2DM can occur rapidly (Elder *et al.*, 2015), there is an evident requirement for satisfactory strategies to reduce postprandial glucose excursions in healthy and at-risk individuals, which may also produce secondary improvements in insulin sensitivity and hypertriglyceridemia (Fava, 2008).

2.5 Strategies to improve metabolic health

2.5.1 Breakfast consumption

One area which has received much attention in recent years is the breakfast meal and the effect of its consumption or omission on energy intake and other health parameters. There does not appear to be a universally accepted definition of breakfast (Betts *et al.*, 2016; Timlin & Pereira, 2007), making comparisons between associational studies difficult. Furthermore, there is no consensus on the optimal composition, timing and amounts of energy and nutrients that define a healthful breakfast (O'Neil *et al.*, 2014). Despite this, evidence from a plethora of cross sectional studies has demonstrated the association between breakfast consumption and reduced incidence of overweight or obesity (Kapantais *et al.*, 2011; Ma *et al.*, 2003; Song *et al.*, 2005) and improved cardiometabolic risk profile (Deshmukh-Taskar *et al.*, 2012; Reutrakul *et al.*, 2014). Moreover, consumption of breakfast appears to be in decline (Kant & Graubard, 2015; Leidy *et al.*, 2016), a trend which has occurred concomitant to the increased global prevalence of obesity (Finucane *et al.*, 2011).

This positive effect of breakfast consumption on obesity and other health parameters is presumed and widely posited by media and public health officials (Brown *et al.*, 2013), however evidence from randomised controlled trials to explain the causality of such associations remains limited. Interactions between breakfast consumption and other behaviours, such as lower levels of smoking and alcohol intake and higher levels of physical activity (Timlin & Pereira, 2007), does not preclude the possibility that residual confounding has occurred.

2.5.1.1 Short term effects on metabolism, appetite and energy intake

It has been proposed that skipping breakfast may be detrimental to appetite control, such that energy intake is higher at subsequent meals. In an acute crossover design, Astbury *et al.* (2011) found that in regular breakfast consumers, energy intake at lunch was 17% greater after skipping breakfast, whilst energy intake over the observation period was not different, indicating full compensation for the omitted breakfast. This is in contrast to the findings of Gonzalez *et al.* (2013), who observed that eating breakfast did not influence energy intake at lunch compared to when breakfast was omitted in healthy participants, despite significantly higher hunger ratings in the no breakfast trial. Levitsky and Pacanowski (2013) reported that intake at lunch was increased following breakfast skipping, however not by enough to fully compensate for the breakfast meal resulting in a net caloric deficit (408 kcal) by the end of the day. A similar effect was observed by Clayton *et al.* (2015), where only partial compensation of energy intake occurred following omission of a standard breakfast, with 24-hour energy intake consequently $19 \pm 5\%$ higher during the breakfast trial. If repeated on a regular basis, the energy deficit created following omission of breakfast could generate weight loss effects, as reported by Geliebter *et al.* (2014) who conducted a 4-week intervention where daily breakfast skipping induced a reduction in body mass (1.18 ± 1.16 kg). In a longer term intervention (16 weeks) conducted in overweight or obese adults who were attempting to lose weight, no difference in weight loss was observed in the breakfast, no breakfast or control (no intervention) groups (Dhurandhar *et al.*, 2014).

Regardless of its impact on body composition, skipping breakfast is likely to be detrimental for insulin sensitivity and glucose tolerance (Dhurandhar, 2016). Compared to the same

period of breakfast omission, 14 days of breakfast consumption resulted in reduced insulin response to a mixed-macronutrient test meal in healthy females (Farshchi *et al.*, 2005), however other measures of insulin sensitivity were not different. This outcome is supported by the findings of Chowdhury *et al.* (2016) who observed reduced post-OGTT insulinaemia in obese adults following 6 weeks of breakfast consumption. No differences in glycaemia were observed in the aforementioned study, however greater glucose variability (Coefficient of variation (CV%): 3.9%, 95% CI: 0.1-7.8%) was detected in the fasting group in the afternoon and evening when the same methods were conducted in lean participants (Betts *et al.*, 2014). Indications that changes in glucose homeostasis may persist over a longer period are supported by Kobayashi *et al.* (2014), who identified increased 24-hour mean glycaemia (recorded using CGM) following omission of breakfast in healthy males. The mechanisms responsible for improvements in subsequent glucose homeostasis are still not fully understood, however it is generally accepted that prior consumption of a carbohydrate load leads to improved glucose tolerance at a later meal, an effect known as the ‘second meal effect’, which is discussed further in section 2.5.1.2.

2.5.1.2 Second meal effect

Many acute nutritional intervention studies investigate the postprandial effects of a single bolus of nutrients, however prior meal consumption can affect the handling of a subsequent meal. Such an effect was first noted almost a century ago by Hamman and Hirschman (1919), who observed that tolerance to a glucose load was improved following prior consumption of glucose. This second meal effect was labelled the ‘Staub–Traugott effect’ after confirmation of these findings by Staub (1921) and Traugott (1922), and is of considerable interest as

postprandial hyperglycaemia has deleterious metabolic effects and is an important risk factor for T2DM and CVD as discussed in section 2.4.3.

Whilst the mechanisms responsible for reduced second meal glycaemia are still not fully understood, likely mediators include changes in NEFA concentration (Tigges & Heer, 2013), as well as altered rates of muscle glycogen synthesis (Jovanovic *et al.*, 2009b) and hepatic glucose production (Bonuccelli *et al.*, 2009). Preprandial NEFA concentration may explain up to half of the variation in postprandial glucose response (Wolever *et al.*, 1995), an affect that may be attributable to the link between elevated NEFA levels and reduced whole-body insulin sensitivity and insulin-stimulated glucose uptake (Boden, 2011). In this context, improved insulin sensitivity is likely to contribute to the Staub–Traugott effect, accentuated by a priming influence of previous exposure on insulin-stimulated processes (Frayn, 2010).

Consumption of pure glucose loads outside of the laboratory setting is uncommon, however the effect of improved glucose tolerance following a second meal appears to be preserved following consumption of mixed-macronutrient meals (Astbury *et al.*, 2011; Jovanovic *et al.*, 2009a; Jovanovic *et al.*, 2009b), enhancing the validity of interpreting such findings in a ‘real world’ context. This is likely to be mediated by a reduction in the rate of gastric emptying following prior consumption of protein (Ma *et al.*, 2009) and fat (Gentilcore *et al.*, 2006). This effect may have implications when considering the efficacy of breakfast consumption on daily glycaemic excursions, and manipulation of breakfast macronutrient content may be an effective strategy to investigate in terms of promoting substantial health benefits.

2.5.2 Protein supplementation

Dietary strategies including modification of complex carbohydrate intake or consuming low glycaemic index (GI) diets may be beneficial in improving metabolic health outcomes (Raben, 2014), however compliance may be difficult to maintain in the long term (Brekke *et al.*, 2004). Modulation of dietary protein intake is an alternative dietary strategy which shows promise in prevention of obesity-related disorders (Astrup *et al.*, 2015). Inadequate protein intake is linked to negative health outcomes including weight gain and loss of skeletal muscle mass (Pasiakos, 2015), which may be particularly detrimental to quality of life in older adults. The World Health Organization (2011) recommends a daily dietary protein intake of $0.83 \text{ g}\cdot\text{kg}^{-1}$ body mass, however it is recognised that intakes above this level may be beneficial for specific populations including athletes (Kreider *et al.*, 2010) and the elderly (Deutz *et al.*, 2014).

Recent evidence associates consumption of higher levels of protein with positive health outcomes beyond just maintenance of muscle mass, with potential implications for the prevention or management of obesity and related co-morbidities. In a cross-sectional analysis, protein intake was inversely associated with BMI and waist circumference, while habitual consumption of protein above recommended levels was associated with higher HDL cholesterol (Pasiakos *et al.*, 2015). This supports the findings of a recent meta-analysis where a small but significant inverse association of protein intake and fat mass was observed in overweight or obese individuals adhering to a higher protein diet for at least 1 year following weight loss (Clifton *et al.*, 2014b).

In addition to evidence linking increased habitual protein consumption with improved body weight management (Astrup *et al.*, 2015; Leidy *et al.*, 2015a; Wycherley *et al.*, 2012), evidence suggests that acute protein ingestion is associated with favourable postprandial responses including greater satiety (Bonnema *et al.*, 2016; Potier *et al.*, 2009), increased postprandial fat oxidation and energy expenditure (Baum *et al.*, 2015; Neumann *et al.*, 2016), with some evidence of improved glycaemia (El Khoury *et al.*, 2014; Park *et al.*, 2015). In addition to the quantity of protein in meals and the wider diet, much interest centres on the impact of protein quality on markers of metabolic health. Protein quality concerns the essential amino acid (EAA) content of a protein in addition to rates of digestion and absorption. Animal proteins are generally considered to be higher quality proteins than plant-based sources, due to increased levels of all EAAs (Pasiakos, 2015). Of these, dairy proteins are perhaps the highest quality source, and an increasing body of evidence is suggestive of a role for such proteins in improving health outcomes (Comerford & Pasin, 2016; Fekete *et al.*, 2016; McGregor & Poppitt, 2013; Ricci-Cabello *et al.*, 2012).

2.5.2.1 Dairy products and proteins

A wealth of epidemiological evidence associates increased dairy consumption with reduced risk of metabolic disease. An inverse relationship has been demonstrated between dairy consumption and glycaemia (Da Silva *et al.*, 2014; Drehmer *et al.*, 2015b) as well as incidence of obesity (Mirmiran *et al.*, 2005; Rautiainen *et al.*, 2016), metabolic syndrome (Drehmer *et al.*, 2015a) and T2DM (Aune *et al.*, 2013; Díaz-López *et al.*, 2016). The relationship between dairy intake and metabolic health is not fully understood however, and although the majority of evidence supports a beneficial effect, this is by no means a universal

observation (Crichton *et al.*, 2011). Struijk *et al.* (2013) found no association between total dairy or consumption of individual dairy products and incidence of T2DM in a prospective analysis of ~6000 Danish adults, whilst Snijder *et al.* (2008) did not detect an association between dairy intake and a number of cardiometabolic risk factors in an elderly Dutch cohort. Such inconsistencies may have arisen due to the considerable heterogeneity of the study designs implemented in this area, where there is extensive variation in study populations, methods of dietary assessment, follow-up duration, diagnostic criteria and control of confounding variables. In addition, many studies report limited information regarding dairy intake criteria, such as the type, quantity and fat content of dairy products included.

The interaction between the various constituents of dairy products also makes delineation of the specific effects of certain components problematic. Milk and other dairy products are complex and diverse foods containing a number of key nutrients and bioactive components which may synergistically contribute to reduction of disease risk (Lovegrove & Givens, 2016), including dairy proteins, oligosaccharides, medium-chain triglycerides, conjugated linoleic acids, calcium and magnesium. It may therefore be prudent to consider the effect of isolated components in randomised controlled trials. In terms of the components mentioned, dairy proteins appear to play a key role in the potential influence of dairy consumption on metabolic regulation, with an accumulating body of evidence supporting the beneficial effects of whey protein consumption in particular (Mignone *et al.*, 2015; Pal & Radavelli-Bagatini, 2013; Zhang *et al.*, 2016).

2.5.2.2 Potential role of whey protein supplementation in metabolic health improvement

Bovine milk protein comprises of two distinct types of proteins, namely whey (~20%) and casein (~80%). Both whey and casein are complete proteins (i.e. they contain all of the EAAs) and have high EAA content, however whey is a considerably richer source of the BCAAs leucine, isoleucine and valine (Hall *et al.*, 2003), which may have important metabolic consequences which shall be discussed further below (section 2.5.2.2.4). Their respective physicochemical properties result in differential effects on gastric emptying and kinetics of digestion and absorption. Casein is precipitated in the stomach by gastric acid resulting in coagulation and slowing of gastric emptying, initiating a small but prolonged postprandial amino acid release (Fekete *et al.*, 2016). In contrast, whey proteins are acid-soluble and are therefore rapidly emptied from the stomach and delivered to the small intestine intact (Pal & Radavelli-Bagatini, 2013), leading to whey being described as a ‘fast protein’ in comparison to the ‘slow protein’ casein (Boirie *et al.*, 1997). This results in a greater increase in amino acids in the plasma which may be important in terms of augmenting the postprandial response, and may be responsible for the greater satiety effect of whey compared to casein proteins (Pal *et al.*, 2014). The proportions of the constituent protein fractions of whey and their various actions are presented in Table 2.3. The hydrolysis of whey protein during digestion releases bioactive peptides which are thought to be responsible for a number of potential functional benefits of whey protein on human health (Adams & Broughton, 2016).

Table 2.3 Composition of whey. Adapted from Pal and Radavelli-Bagatini (2013), Krissansen (2007) and Sousa *et al.* (2012).

Constituent	Approximate Proportion (% of Energy)	Actions
β -Lactoglobulin	35	Has high branched chain amino acid (~25%) content. Captures hydrophobic molecules, participating in the reduction of intestinal absorption of lipids.
α -Lactalbumin	12	Has highest content of tryptophan (6%) of all dietary proteins. It is rich in lysine, leucine, threonine, and cysteine. It has the ability to bind to minerals such as Ca and Zn, positively affecting their absorption.
Glycomacropeptide	12	Formed from the digestion of κ -casein during coagulation of cheese. It is high in EAAs, particularly BCAAs.
Proteose peptone 3	12	Foaming and emulsifying properties.
Immunoglobulin	8	Four classes of immunoglobulins are present: IgG, IgA, IgM, and IgE. It functions as an antioxidant and increases immunity.
Bovine serum albumin	5	Good amino acid profile with the function of binding to lipids.
Lactoferrin	1	Inhibits the production of pro-inflammatory cytokines and protects against the development of hepatitis.
Lactoperoxidase	0.5	Important antimicrobial properties
Other components (lactose) and minor proteins	~15	
Vitamins and minerals (Ca, Fe, K, P, Mg, Zn)	Variable	
Traces of milk fat	Variable	

Ca, calcium; BCAA, branched-chain amino acid; EAA, essential amino acid; Fe, iron; K, potassium; Mg, magnesium; P, phosphorus; Zn, zinc.

The number of published acute studies investigating the effects of whey protein consumption on postprandial responses has increased considerably in recent years. A summary of these studies in normal-weight, overweight/obese and T2DM populations is provided in Table 2.4, followed by discussion of these effects, potential mechanisms and limitations of the current body of research.

Table 2.4 Summary of acute randomised trials investigating the effect of whey protein consumption on metabolic and appetite responses.

Reference	Participants	Design	Treatment	Comparison	Key Outcomes
<u>NORMAL-WEIGHT PARTICIPANTS</u>					
Abou-Samra <i>et al.</i> (2011)	Healthy: males ($n=32$) Age: 25 ± 4 years BMI: $24.0 \pm 0.4 \text{ kg}\cdot\text{m}^{-2}$	Randomised, single-blind, crossover design. Six sessions scheduled at least 2 days apart. Six preloads (20 g added to 250 ml water) served 30 minutes prior to ad libitum breakfast meal.	Whey protein (20 g)	- Pea protein - Casein - Egg albumin (all matched for protein) - Maltodextrin (matched for energy) - Control (water)	- Food intake was lower only after casein and pea protein compared to water. - Feelings of satiety were significantly higher after casein and pea protein compared to other preloads. - Blood glucose response was lower after whey compared to other preloads.
Akhavan <i>et al.</i> (2010)	Healthy: males ($n=16$) Age: 22 ± 1 years BMI: $22.6 \pm 0.4 \text{ kg}\cdot\text{m}^{-2}$	Randomised, crossover design. Participants consumed preloads with 300 ml water. After 30 minutes:	Experiment 1: Whey protein (10, 20, 30, 40 g)	Experiment 1: - Control (isovolumetric flavoured water)	Experiment 1: - 20-40g whey protein suppressed food intake and 10-40g reduced post-meal blood glucose concentration and AUC.
	Healthy: 9 females, 12 males ($n=21$) Age: 22 ± 1 years BMI: $22.1 \pm 0.5 \text{ kg}\cdot\text{m}^{-2}$	Experiment 1: <i>Ad libitum</i> pizza meal Experiment 2: Standard pizza meal	Experiment 2: Whey protein (5, 10, 20, 40 g)	Experiment 2: - Control (isovolumetric flavoured water) - Whey protein hydrolysate (10 g)	Experiment 2: - 10-40g whey protein, but not whey protein hydrolysate, reduced post-meal BG AUC and insulin AUC in a dose-dependent manner.
Akhavan <i>et al.</i> (2014)	Healthy: males ($n=10$) Age: 18-29 years BMI: $18.5\text{-}29.4 \text{ kg}\cdot\text{m}^{-2}$	Randomised, single-blind, crossover design. Participants consumed preloads with 300 ml water. Standardised pizza meal eaten 30 minutes after preload.	Whey protein (10, 20 g)	- Glucose (10, 20 g) - Control (isovolumetric flavoured water)	- Whey protein slowed pre-meal gastric emptying vs control and 10g glucose. - Both whey protein and glucose (10 and 20 g) reduced post-meal glycaemia with similar CCK, amylin, ghrelin and GIP responses. - For total duration (0-230 minutes), whey protein resulted in lower mean plasma glucose, insulin and C-peptide but higher GLP-1 and PYY compared to glucose preloads.
Anderson <i>et al.</i> (2004)	Healthy: males ($n=13$) Age: 22 ± 3 years BMI: $22.1 \pm 0.7 \text{ kg}\cdot\text{m}^{-2}$	Randomised, single-blind, crossover designs. Participants consumed test drinks as 400 ml preloads.	Experiment 1: Whey protein ($0.65\text{g}\cdot\text{kg}^{-1}$ body mass)	- Sucrose - Egg albumin - Soy protein (all isoenergetic) - Control (water)	- Compared to control, preloads (45-50g) of whey and soy protein, but not egg albumin, suppressed food intake at a pizza meal consumed 1 hour later. - Suppression of food intake after whey protein, consumed as intact protein or peptides, extended to 2 hours.
	Healthy: males ($n=22$) Age: 22 ± 1 years BMI: $22.8 \pm 0.4 \text{ kg}\cdot\text{m}^{-2}$	An <i>ad libitum</i> pizza buffet meal was served 60 minutes post-preload in experiments 1 and 2, and 120 minutes post-preload in experiment 3.	Experiment 2: Whey protein (50 g)	- Egg albumin (isoenergetic) - Control (water)	
	Healthy: males ($n=10$) Age: 18-35 years BMI: $20\text{-}27 \text{ kg}\cdot\text{m}^{-2}$		Experiment 3: Whey protein (50 g)	- Whey protein hydrolysate (10 g) - Control (water)	
Astbury <i>et al.</i> (2010)	Healthy: 12 females, 12 males ($n=24$) Age: 30 ± 9 years BMI: $23.0 \pm 2.4 \text{ kg}\cdot\text{m}^{-2}$	Randomised, single-blind, crossover designs. Participants completed 4 visits in each study, separated by at least 3 days.	Experiment 1 (all 1675kJ): Whey protein (12.9g) 12.5% of energy Whey protein (25.4g) 25% Whey protein (50.4g) 50%	Experiment 1: - Control (isovolumetric flavoured water)	Experiment 1: - Energy intake after control was significantly higher than after test preloads. - Intake following 12.5% protein was significantly higher than after 25% and 50% preloads. - There were no differences in subjective appetite ratings between preloads.
	Healthy: 13 females, 13 males ($n=26$) Age: 25 ± 5 years BMI: $22.8 \pm 2.5 \text{ kg}\cdot\text{m}^{-2}$	Liquid preloads (400 ml) were provided, followed 90 minutes later by <i>ad libitum</i> pasta meal.	Experiment 2 (all 1047kJ): Whey protein (6.8g) 10% of energy Whey protein (13.1g) 20% Whey protein (25.4g) 40%	Experiment 2: - Control (isovolumetric flavoured water)	Experiment 2: - Energy intake after control was significantly higher than after test preloads, with no difference in energy intake between protein containing preloads. - There were no differences in subjective appetite ratings between preloads.
Chungchunlam <i>et al.</i> (2015)	Healthy: females ($n=18$) Age: 22 ± 1 years BMI: $22.7 \pm 0.4 \text{ kg}\cdot\text{m}^{-2}$	Randomised, single-blind, crossover design. Four visits separated by at least 3 days. Liquid preloads (300 ml) consumed at lunchtime on each occasion followed after 120 minutes by <i>ad libitum</i> fried rice meal.	Whey protein (45 g)	- Maltodextrin (45 g) (Energy matched)	- Energy intake was reduced following whey protein. - No effect of preload on subjective appetite responses. - Total amino acid and BCAA concentration were increased after whey protein.
Gunnerud <i>et al.</i> (2012a)	Healthy: 3 females, 6 males ($n=9$) Age: 22-30 years BMI: $25.8 \pm 3.4 \text{ kg}\cdot\text{m}^{-2}$	Randomised, crossover design. Five visits separated by at least 2 days. Test meals served as breakfast on each occasion. Samples taken 120 minutes postprandially.	Whey protein (16.2 g) (with 25 g lactose)	- White wheat bread (control) - Casein (16.8 g) - Bovine milk - Human milk (all carbohydrate matched)	- Glucose iAUC was reduced after all protein meals compared to control. - The insulin response was significantly higher after whey protein. - Early phase plasma amino acids were correlated to insulin and incretin secretion.

Reference	Participants	Design	Treatment	Comparison	Key Outcomes
Gunnerud <i>et al.</i> (2012b)	Healthy: 9 females, 5 males ($n=14$) Age: 20-28 years BMI: $21.9 \pm 0.6 \text{ kg}\cdot\text{m}^{-2}$	Randomised, single-blind, crossover design. Seven visits separated by ~7 days. Fed standard breakfast (ham sandwich – 50 g carbohydrate, 13 g protein) with different drinks (served immediately pre-meal). Samples taken 180 minutes postprandially.	Whey protein (9 g)	- Whey protein +5 amino acids - Whey protein +6 amino acids - Soy protein - Soy protein +5 amino acids - Soy protein +6 amino acids (all energy matched) - Control (water)	- All protein drinks reduced the glycaemic response in the first 60 minutes. - No differences for insulinaemic indices. - The early insulin response (0-15min iAUC) correlated positively with plasma amino acids, GLP-1, GIP and glycaemic profile.
Gunnerud <i>et al.</i> (2013)	Healthy: 7 females, 5 males ($n=12$) Age: 20-23 years BMI: $22.9 \pm 0.7 \text{ kg}\cdot\text{m}^{-2}$	Randomised, single-blind, crossover design. Four visits separated by ~7 days. Different test drinks (250 ml) served as breakfast on each occasion. Samples taken 120 minutes postprandially.	Whey protein (4.5, 9, 18 g) (combined with 25 g glucose)	- Control (water + 25 g glucose)	- Linear dose-response relationships found between whey protein supplementation and postprandial glycaemia, insulinaemia and amino acids. - 18 g and 9 g whey protein significantly reduced glycaemia. - 18 g increased insulin response. - Amino acids responded in dose-dependent manner.
Hursel <i>et al.</i> (2010)	Healthy: 18 females, 17 males ($n=35$) Age: 21 ± 2 years BMI: $23.0 \pm 2.1 \text{ kg}\cdot\text{m}^{-2}$	Randomised, single-blind, crossover design. Three visits separated by ~7 days. Test breakfasts served and samples taken 240 minutes postprandially.	Whey protein yoghurt drink, 41% energy from protein	- Control (normal protein yoghurt drink), 15% energy from protein - α -lactalbumin-enriched whey protein yoghurt drink, 41% energy from protein (all matched for energy)	- Both high protein breakfasts increased thermogenesis more than normal protein. - α -lactalbumin suppressed hunger more than whey or control.
Hutchison <i>et al.</i> (2015)	Healthy: males ($n=18$) Age: 25 ± 1 years BMI: $21.6 \pm 0.5 \text{ kg}\cdot\text{m}^{-2}$	Randomised, double-blind, crossover design. Three visits separated by 7-11 days. Test drinks (~450 ml) served and samples taken 180 minutes postprandially, followed by <i>ad libitum</i> buffet meal.	Whey protein (30 g) Whey protein (70 g) Matched for osmolality	- Control (saline) Matched for osmolality	- Gastric emptying was comparable between protein drinks. - Energy intake was suppressed similarly by both protein loads. - Glucose was modestly reduced following the high whey protein load.
Nilsson <i>et al.</i> (2007)	Healthy: 6 females, 6 males ($n=12$) Age: 20-30 years BMI: $22.4 \pm 0.6 \text{ kg}\cdot\text{m}^{-2}$	Randomised, crossover design. Five visits separated by at least 1 week. Drinks (250 ml) were provided as breakfast. Postprandial blood samples taken for 120 minutes.	Whey protein (18 g) (combined with 25 g glucose) - Control (25 g glucose)	- Lysine and threonine - Leucine, isoleucine and valine - Leucine, isoleucine, valine, lysine and threonine (all combined with 25 g glucose) - Control (25 g glucose)	- Drink with leucine, isoleucine, valine, lysine and threonine mimicked the glycaemic and insulinaemic responses seen after whey ingestion. - Consumption of whey resulted in 56% smaller glucose AUC and 60% larger insulin AUC than reference drink. - Whey drink gave 80% greater GIP response, whereas drinks containing free amino acids did not significantly affect GIP secretion.
Silva Ton <i>et al.</i> (2014)	Healthy: 6 females, 4 males ($n=10$) Age: 25 ± 1 years BMI: $22.0 \pm 0.8 \text{ kg}\cdot\text{m}^{-2}$	Randomised, crossover design. Four visits separated by at least 1 day. Test drinks consumed as preloads 30 minutes prior to consumption of 25 g glucose. Postprandial blood samples taken for 120 minutes.	Whey protein ($0.5\text{g}\cdot\text{kg}^{-1}$ body mass)	- Control (isovolumetric flavoured water) - Soy protein ($0.5 \text{ g}\cdot\text{kg}^{-1}$ body mass) - Egg albumin ($0.5\text{g}\cdot\text{kg}^{-1}$ body mass)	- Whey and soy protein reduced glucose iAUC by 56.5% and 44.4% compared to control, respectively.
NORMAL-WEIGHT AND OVERWEIGHT/OBESE PARTICIPANTS					
Bowen <i>et al.</i> (2006a)	Healthy (25) and overweight (47): males ($n=72$) Age: 55 ± 3 years BMI: Healthy – 23.3 ± 0.2 , obese – $30.1 \pm 0.5 \text{ kg}\cdot\text{m}^{-2}$	Randomised, single-blind, crossover design. Four visits separated by 7 days. Liquid preloads (450 ml) consumed on each occasion followed after 180 minutes by <i>ad libitum</i> buffet meal.	Whey protein (50 g)	- Soy (50 g) - Gluten (50 g) - Glucose (63 g) control (All energy matched)	- Energy intake was equally reduced after whey, soy or gluten: all 10% lower than after glucose in both lean and obese. - Fasting and postprandial GLP-1 was higher in overweight participants.
Hoefle <i>et al.</i> (2015)	Healthy (15) and prediabetes (15): 5 females, 25 males ($n=30$) Age: Healthy – 26 ± 1 , prediabetes – 62 ± 2 years BMI: Healthy – 23.9 ± 0.5 , prediabetes – $29.0 \pm 1.5 \text{ kg}\cdot\text{m}^{-2}$	Randomised, single-blind, crossover design. Three visits separated by at least 2 days. Test drinks served and samples taken 240 minutes postprandially.	Whey protein (50 g) (combined with 50 g maltodextrin)	- Control (50 g maltodextrin) - Casein (50 g + maltodextrin 50 g)	- Whey protein and casein similarly reduced postprandial glucose excursions in healthy and prediabetic participants. - Both proteins increased plasma insulin despite simultaneous increases in glucagon concentrations.
Zafar <i>et al.</i> (2013)	Healthy: females ($n=12$) Age: 22 ± 5 years BMI: $22.6 \pm 2.4 \text{ kg}\cdot\text{m}^{-2}$ Healthy (15) and overweight (15): females ($n=30$) Age: 22 ± 5 years BMI: Healthy – 21.5 ± 2.1 , overweight – $30.7 \pm 2.5 \text{ kg}\cdot\text{m}^{-2}$	Randomised, crossover design. Four visits scheduled ~1 week apart. Sweetened beverages (300 ml) consumed followed after 180 minutes by <i>ad libitum</i> pizza meal. Randomised, crossover design. Three visits scheduled ~1 week apart. Sweetened beverages (300 ml) consumed, samples taken for 120 minutes.	Whey protein (25 g)	- Whey protein (25 g) + glucose (50 g) - Glucose (50 g) - Glucose (75 g) - Whey protein (25 g) + glucose (25 g) - Glucose (25 g)	- Peak blood glucose and iAUC was reduced after whey protein and whey protein supplemented glucose in both normal weight and overweight participants. - Energy intake was reduced after both WP drinks, with or without glucose.

Reference	Participants	Design	Treatment	Comparison	Key Outcomes
OVERWEIGHT/OBESE PARTICIPANTS					
Bowen <i>et al.</i> (2006b)	Overweight: males ($n=19$) Age: 53 ± 6 years BMI: 32.1 ± 3.7 kg·m ⁻²	Randomised, single-blind, crossover design. Four visits separated by 7 days. Liquid preloads consumed on each occasion followed after 180 minutes by <i>ad libitum</i> buffet meal.	Whey protein (55 g)	- Casein (55 g) - Lactose (56 g) - Glucose (56 g) control (matched for energy)	- Acute appetite and energy intake were equally reduced after whey, casein or lactose, all 10% lower than after glucose. - CCK was 71% higher 90 minutes after protein preloads compared to glucose and lactose, which predicted appetite at 180 minutes. - There was a small increase in BCAAs after whey compared to casein, but this was independent of appetite and energy intake.
Bowen <i>et al.</i> (2007)	Obese: males ($n=28$) Age: 57 ± 2 years BMI: 32.5 ± 0.6 kg·m ⁻²	Randomised, double-blind, crossover design. Four visits separated by 7 days. Liquid preloads consumed on each occasion followed after 240 minutes by <i>ad libitum</i> buffet meal.	Whey protein (50 g)	- Fructose (25 g) + Whey protein (25 g) - Fructose (50 g) - Glucose (50 g) control (matched for energy)	- There was no difference in energy intake between the preloads. - Whey protein produced a prolonged suppression of ghrelin and elevations of GLP-1 and CCK that were reduced when combined with fructose.
Pal <i>et al.</i> (2010b)	Overweight and obese: females ($n=20$) Age: <66 years BMI: 25-40 kg·m ⁻²	Randomised, single-blind, crossover design. Three visits separated by 1 week. Test drinks (~400 ml) consumed alongside mixed macronutrient breakfast and postprandial blood samples taken for 360 minutes.	Whey protein (45 g)	- Sodium caseinate (45 g) - Control (glucose 45 g) (matched for energy)	- Appearance of triglycerides in the blood decreased after the whey meal compared to control and casein by 21% and 27% respectively. - Glucose was reduced after whey and casein compared to control. - There was a reduction in the AUC for triglyceride:ApoB48 ratio after whey compared to glucose and casein, reflecting reduced exposure to triglyceride-rich lipoproteins.
Petersen <i>et al.</i> (2009)	Obese: 3 females, 7 males ($n=10$) Age: 44 ± 9 years BMI: 33.6 ± 4.8 kg·m ⁻²	Randomised, crossover design. Visits separated by at least 1 day. Drinks (250 ml) were provided as breakfast. Postprandial blood samples taken for 120 minutes.	5, 10, 20 g Intact whey protein + peptides (all combined with 50 g glucose)	- Control (50 g glucose)	- Increasing doses of intact whey and peptides decreased blood glucose iAUC in a dose-dependent manner.
T2DM PARTICIPANTS					
Clifton <i>et al.</i> (2014a)	Prediabetes (11) and T2DM (13): 11 females, 13 males ($n=24$) Mean age: 60 years Mean BMI: 32 kg·m ⁻²	Randomised, crossover design. Four visits, including 2 test days and 2 placebo days; data from both days was averaged. Liquid preloads (150 ml) followed after 15 minutes by standardised carbohydrate-based breakfast.	Whey protein (17 g) with 5 g added fibre (guar)	- Control (water)	- Peak glucose was reduced by 2.1 mmol·l ⁻¹ at 45 min. - Mean glucose over 3 hours was reduced by 0.8 mmol·l ⁻¹ . - There was no difference between those with diabetes or prediabetes or those on medication or not on medication.
Frid <i>et al.</i> (2005)	T2DM: 6 females, 8 males ($n=14$) Age: 27-69 years BMI: 26.2 ± 3.1 kg·m ⁻²	Randomised, crossover design. Two visits separated by at least 7 days. High GI breakfast (white bread) and lunch (mashed potato and meatballs) 4 hours post breakfast, were served on both occasions, with trial-dependent addition of whey or ham and lactose.	Whey protein (18.2 g) with both meals = 36.4 g in total	- Ham and lactose (matched for protein and carbohydrate content)	- Insulin AUC was 31% higher after breakfast and 57% higher after lunch when whey was included. - Glucose AUC was similar after breakfast but reduced by 21% after lunch with whey protein ingestion. - No difference in GLP-1 after both meals. - GIP response was higher after whey ingestion.
Jakubowicz <i>et al.</i> (2014)	T2DM: 6 females, 9 males ($n=15$) Age: 64 ± 1 years BMI: 26.7 ± 1.2 kg·m ⁻²	Randomised, crossover design. Test drinks (250 ml) consumed as a preload 30 minutes prior to a high GI breakfast and samples taken 180 minutes postprandially.	Whey protein (50 g)	- Control (water)	- Blood glucose levels were reduced by 28% after whey protein preload, with a uniform reduction in early and late phases. - Insulin and GLP-1 responses were significantly higher following whey protein. - DPP-IV activity was not different between trials.
Ma <i>et al.</i> (2009)	T2DM: 7 females, 1 males ($n=8$) Age: 58 ± 3 years BMI: 28.6 ± 1.3 kg·m ⁻²	Randomised, crossover design. Preload administered 30 minutes before mashed potato + glucose meal (59.1 g carbohydrate). Whey protein added to preload, meal, or not added.	Whey protein in preload (55 g) Whey protein in meal (55 g)	- Control	- Gastric emptying was slowest after the whey preload, and slower after whey in meal than control. - Glucose iAUC reduced for whey preload and whey meal compared to control. - GIP, insulin and CCK were higher during both whey trials than control. - GLP-1 was only higher after the whey preload.

ApoB48, apolipoprotein-B48; AUC, area under the curve; BCAA, branched-chain amino acids; BMI, body mass index; CCK, cholecystokinin; DPP-IV, dipeptidyl peptidase IV; GI, glycaemic index; GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-like peptide-1; iAUC, incremental area under the curve; PYY, peptide tyrosine-tyrosine; T2DM, type 2 diabetes mellitus.

2.5.2.2.1 Effect on glycaemia

Studies in a variety of populations have identified the capacity of whey protein to augment the postprandial insulinaemic response, with potential concomitant effects on glycaemia. Studies with overweight or T2DM populations are fewer in number than those with healthy sample groups, and it remains unclear whether the magnitude of the insulinotropic effect following whey supplementation is sufficient to consistently reduce postprandial glycaemia in insulin resistant individuals, where hyperinsulinaemia may be commonplace. When lean ham and lactose were substituted by the equivalent amount (18.2 g) of whey protein in high GI meals given to individuals with T2DM, the insulin response was significantly increased following both breakfast (18%) and lunch (49%), however glycaemia was similar following the breakfast meal, and only reduced after lunch (21%) (Frid *et al.*, 2005). It is possible that the post-breakfast increase in circulating insulin was not sufficient to overcome the greater insulin resistance observed in the postabsorptive state (Plat *et al.*, 1996). The post-lunch reduction in glycaemia was not insignificant however, and is comparable with the 28% reduction in glucose area under the curve (AUC) observed alongside a simultaneous 105% increase in insulin AUC by Jakubowicz *et al.* (2014). This study compared 50 g whey protein consumption with a control (water) drink before a high GI breakfast in participants with T2DM. The higher dose of protein and the fact that it was administered as a preload 30 minutes prior to the breakfast meal in the latter study, in addition to the fact that it was a single meal test, means that these findings are not directly comparable. Despite this, the effect observed on postprandial glycaemia in both studies was greater than that detected over the same time period (180 minutes) following administration of nateglinide, a pharmacologic rapid-acting insulin secretagogue (Gribble *et al.*, 2001), emphasising the potential of dietary

strategies for glycaemic management in individuals with impaired glucose metabolism. In a further study in participants with T2DM, similar reductions in post-meal glycaemia were observed whether 55 g whey was given before (30 minutes) or alongside a high GI meal compared with no supplementation (Ma *et al.*, 2009). Rate of gastric emptying was also measured as part of this protocol and was inhibited by the presence of whey, however there was no evidence that the difference in emptying rates between the whey preload and whey in meal trials was reflected in postprandial glycaemia.

A limited number of studies have assessed the glycaemic response following whey supplementation in non-diabetic overweight or obese populations. A significant reduction in glucose AUC was observed over 180 minutes following consumption of a 55 g liquid protein preload in comparison to lactose and glucose controls in overweight or obese men (BMI ranging from 26.8 to 40.4 kg·m⁻²), however changes in insulin concentrations were not affected by condition (Bowen *et al.*, 2006b). Data for whey and casein protein preloads were pooled together however, and the differential effects of whey and casein supplementation on a variety of markers (Boirie *et al.*, 1997; Hall *et al.*, 2003) makes comparison with other whey supplementation protocols difficult. Nevertheless, the findings of Pal *et al.* (2010b) support the observations described above. This group reported a 16% reduction in post-meal glycaemia over 360 minutes in a group of overweight or obese post-menopausal females following 45 g whey protein compared to an energy-matched glucose control. Similarly to above, this intervention did not influence post-meal insulinaemia, indicating that milk-derived proteins may influence plasma glucose via insulin-independent mechanisms or via temporal changes in insulin sensitivity. Key differences exist between the design of these studies however, which may influence any direct comparisons. Pal *et al.* (2010b)

administered protein alongside a mixed macronutrient breakfast meal, simulating the effect of consuming a realistic breakfast meal (bread roll with margarine and spread) with additional protein, which may be a more ecologically valid method than the provision of a test beverage only (Bowen *et al.*, 2006b). The control arm did consist of 45 g glucose however, therefore a direct effect of adding protein to the meal in comparison with adding no additional nutrients cannot be established.

Only one acute study has focussed on the glycaemic response to whey protein in both normal-weight and overweight participants. Zafar *et al.* (2013) investigated the effects of 25 g whey protein or glucose, or a combination of both (50 g), on postprandial glycaemia for 120 minutes in 15 normal-weight and 15 overweight young females. Whey protein was effective in reducing postprandial glycaemia compared to glucose and glucose with whey, while the latter condition suppressed blood glucose compared to control. Glucose AUC was 16% higher following the pure glucose drink in overweight compared to normal-weight participants, however this disparity in glucose tolerance between groups was corrected when whey protein was administered, with glucose attenuated to similar levels in both groups following whey protein beverages. The authors hypothesise that such an effect may be due to an enhancement of insulin sensitivity by whey protein in overweight participants, however insulin concentrations were not assessed in this study, making this a speculative assumption. Such an effect has, however, been observed following chronic whey protein consumption in both rats (Belobrajdic *et al.*, 2004) and humans (Pal *et al.*, 2010a).

A dose-response relationship between whey protein load and post-meal glucose reduction has been observed in a number of studies in normal weight (Akhavan *et al.*, 2010; Gunnerud

et al., 2013) and obese populations (Petersen *et al.*, 2009). Both studies in lean healthy individuals observed a linear effect of glucose reduction with glucose loads ranging from 5-40 g (Akhavan *et al.*, 2010) and 4.5-18 g (Gunnerud *et al.*, 2013), however differential patterns in insulin response were reported between studies. In the former, post meal insulin was reduced in a dose-dependent manner, however when pre-meal (post-preload) values were included there was no difference between conditions in cumulative insulin AUC. In the latter, a dose-dependent increase in insulin AUC was reported, with all whey doses resulting in higher insulin AUC compared to control, albeit only significantly higher following the highest dose (18 g). Such a contrast may be the result of a number of fundamental differences in study design, since Akhavan *et al.* (2010) administered whey as a preload 30 minutes before consumption of a mixed-macronutrient pizza meal, as a standalone supplement in flavoured water, whereas Gunnerud *et al.* (2013) did not use a preload design, and administered whey as a breakfast beverage combined with 25 g glucose. The addition of 18 g whey to 25 g glucose has previously been investigated by the same group and found to significantly reduce glycaemia with a concomitant increase in insulin AUC (Nilsson *et al.*, 2007), and it has been proposed that amino acid availability may potentiate the increased insulin response. This is an attractive theory since plasma amino acids also increased in a dose-dependent manner in this study, while a previous investigation revealed that a combination of whey and free amino acids induced a rapid insulinogenic effect which influenced early glycaemia (Gunnerud *et al.*, 2012b). Plasma amino acids were not measured by Akhavan *et al.* (2010), however they argue that insulin alone cannot be the only cause of reduced post-meal glycaemia since they observed such reductions in the presence of reduced post-meal and similar cumulative insulin concentrations.

2.5.2.2.2 Effect on lipid metabolism

Fasting levels of blood lipids are commonly used in the clinical setting as a markers of CVD risk, however the importance of the postprandial milieu, and of post-meal hypertriglyceridaemia in particular, as a determinant of CVD risk is increasingly being recognised by researchers (Kolovou *et al.*, 2011). Evidence from acute trials assessing the effect of whey protein on markers of lipaemia is limited and has shown mixed results.

The effects of 45 g of whey, casein or glucose served alongside a mixed macronutrient meal on glycaemia were described above, however the study by Pal *et al.* (2010b) was primarily designed to investigate effects on lipaemic markers in overweight and obese females. No condition-dependent differences were detected for total, LDL or HDL cholesterol, NEFA or ApoB48, however triglyceride AUC was reduced by 21% and 27% following whey consumption compared to glucose and casein respectively. In addition AUC for triglyceride:ApoB48 ratio was significantly reduced following whey protein compared to casein and glucose, reflecting decreased exposure to smaller triglyceride-rich chylomicron particles. This is currently the only acute study that has compared whey protein consumption with a non-protein control in the context of investigating postprandial lipaemia, however other studies have compared the same 45 g dose with different protein types or whey protein fractions. Mortensen *et al.* (2009) compared the effects of whey with casein and non-dairy proteins (cod and gluten) alongside a high fat meal on postprandial lipaemia in participants with T2DM. Similarly to above, whey protein reduced triglyceride AUC in comparison to casein, and also appeared to outperform other proteins in terms of modulating the triglyceride

response, in addition to promoting greater suppression of plasma NEFA for 6 hours postprandially.

In contrast to the findings above, Mariotti *et al.* (2015) reported that triglyceride AUC was increased following consumption of whey or α -lactalbumin-enriched whey protein compared with casein protein alongside a high fat meal. Such a discrepancy may partly be explained by the different populations. Overweight but otherwise healthy young men were included in this study, whereas the population in the study of Pal *et al.* (2010b) consisted of post-menopausal women and Mortensen *et al.* (2009) included 12 T2DM participants with mean age of 65 years. Nine of that group were taking lipid-lowering medication which would likely interrupt lipoprotein and fatty acid metabolism compared to healthy volunteers. In addition, the protein was not completely mixed with the other macronutrients in the test meal served in the above two studies, whereas Mariotti *et al.* (2015) served a homogenised test drink containing cream, sucrose and the supplemental protein. This may have had implications for nutrient interaction during digestion.

Notwithstanding this, in a study from the same group using an identical test meal as Mortensen *et al.* (2009), in an obese non-diabetic population, postprandial plasma triglyceride concentrations were more favourable following the addition of whey to the meal (Holmer-Jensen *et al.*, 2013). Triglyceride AUC was 61% higher after cod protein and 66% higher after gluten protein, although values were not different compared to casein after 6 hours. Whey and casein also showed significantly greater suppression of NEFA during this period, although this was not significant over the full 8-hour postprandial period.

Current evidence provides a mixed picture regarding the potential of whey protein to improve markers of dyslipidaemia. Studies have shown that measures of HDL and LDL cholesterol tend to remain stable following consumption of dairy proteins with high fat meals, however there is evidence that whey may have important influences on other circulating lipids. The mechanisms underpinning these interactions are currently not well understood however, and further studies are required to investigate the effects that whey protein may have when consumed with test meals that better reflect those consumed in daily living, in both lean and overweight individuals.

2.5.2.2.3 Effect on satiety and energy intake

Whilst it is generally accepted that protein ingestion can strongly influence satiety and food intake (Potier *et al.*, 2009), the type of protein may be an important factor in determining the magnitude of this effect. Whey protein may increase satiety (Veldhorst *et al.*, 2009) and reduce food intake (Hall *et al.*, 2003) compared to casein, and has also been shown to produce favourable effects on hunger and energy intake compared to non-dairy proteins in some studies (Pal & Ellis, 2010), but not others (Abou-Samra *et al.*, 2011). In normal-weight young males, preloads (45-50 g) of whey and soy protein, but not egg, reduced ad libitum energy intake at a pizza buffet meal 1 hour later compared to a water control (Anderson *et al.*, 2004). In contrast Bowen *et al.* (2006a) found that although energy intake was reduced by 10% 180 minutes post ingestion of 50 g whey compared with glucose control, this was not different from soy and gluten preloads, and the same effect was observed in both lean and obese participants. There was also no difference in subjective measures of hunger and prospective food consumption between trials or by participant weight status. It is speculated that obese

participants may have reduced sensitivity to the appetite suppressing effects of GLP-1, as postprandial levels of this hormone were higher in the obese group, without affecting energy intake. The effect on behavioural responses of mixing groups of lean and obese participants during the *ad libitum* buffet meal may also be responsible for the lack of difference between participant groups.

Another study by the same group in overweight and obese participants reported that *ad libitum* energy intake was reduced by 493 kJ (~10%) at a buffet meal 180 minutes following consumption of 55 g whey protein compared to an energy-matched glucose control, however this reduction was similar to that observed for lactose and casein preloads (Bowen *et al.*, 2006b), with a similar effect observed in average appetite ratings. In a different design, there were no differences in subjective appetite responses and similar amounts of energy were consumed at a buffet meal 4 hours post ingestion of 50 g preloads of whey, fructose, a combination of these ingredients, or glucose (Bowen *et al.*, 2007). This was despite the fact that whey produced contrasting post-ingestion profiles of ghrelin, CCK and GLP-1, an effect that was attenuated when 50% of the whey was replaced by fructose. It could be speculated that obese individuals may require greater doses of whey protein to promote satiety, as similar amounts to those administered in this studies have produced positive effects on satiety in normal-weight individuals (Pal & Ellis, 2010), indeed there is evidence of a dose-response relationship in such individuals, with multivariate analysis predicting a 24 kJ reduction in subsequent energy intake for every 1 g increase in whey protein content of the preload.

Astbury *et al.* (2010) reported stepwise reductions in lunchtime energy intake with increasing whey protein content of mixed macronutrient preloads served 90 minutes prior to the meal

in 24 healthy young adults. Consumption of a 1675 kJ preload with whey protein constituting 12.5% of energy resulted in reduced food intake when compared to control (0 kJ), but greater intake than after 25% and 50% preloads. Interestingly there were no differences in subjective appetite responses between trials in this study, indicating that adjustments in preload macronutrient composition may have resulted in physiological changes that were sufficient to reduce energy intake without affecting subjective feelings. The lack of physiological response data makes speculation of such mechanisms difficult, however. In another dose-response study, preloads containing doses of whey above 10 g (20-40 g) were adequate in reducing energy intake at a subsequent *ad libitum* pizza buffet meal in healthy young males, with the largest reduction occurring after a 40 g protein preload (Akhavan *et al.*, 2010). The fact that the lowest preload dose, which is comparable to the lowest dose in the above study, did not significantly augment subsequent intake may be a result of the smaller time period (30 minutes) between preload and buffet meal. This may also have influenced the degree of energy compensation at the meal, as cumulative intake (preload+meal) was not different between trials. Notwithstanding this, the 78 kcal reduction observed following the 10 g preload was predicted to be significant if the sample size was increased to 40 participants, therefore statistical power may also have been an issue. Similarly to above, there were no effects of condition on subjective appetite ratings prior to the meal. In contrast, evidence of a dose-response effect on energy intake is not supported by the similar reduction in intake observed 180 minutes post ingestion of 30 g and 70 g whey preloads in healthy males (Hutchison *et al.*, 2015). The use of a 70 g dose represents a load over and above that which may be obtained from usual serving sizes however. Taken together with the evidence from previous studies (Akhavan *et al.*, 2010; Astbury *et al.*, 2010), these findings are indicative of ~30-40 g protein representing a threshold after which greater loads do not result in further

suppression in subsequent intake. This may be a result of plasma amino acids exceeding a threshold level (Veldhorst *et al.*, 2009).

When studies have assessed the impact of protein content rather than type (i.e. a non-protein comparator was used), findings have generally confirmed the satiating effect of protein consumption. A 45 g whey protein preload drink reduced food intake after 120 minutes in comparison to an energy-matched maltodextrin preload in healthy females (Chungchunlam *et al.*, 2015), however differences in subjective appetite were not apparent. Addition of 25 g whey protein to 50 g glucose reduced energy intake at a pizza buffet meal 180 minutes later by 21% (279 kcal) in healthy young females (Zafar *et al.*, 2013), although differences in subjective appetite were only detected at the 180 minute time point. When the energy content of the preload is taken into account, intake was still 11% lower after addition of whey, although this finding was not statistically significant. This does however, emphasize the potential for investigating supplementation strategies whereby whey protein is an addition to a meal or preload, rather than a substitution for other nutrients, which may be a more realistic approach for individuals to adhere to.

2.5.2.2.4 Potential mechanisms underlying the effects of whey protein supplementation

It is likely that several interconnected mechanisms interact to affect postprandial responses, as depicted in Figure 2.2, with evidence suggesting that whey lowers glucose via insulin-dependent and insulin-independent mechanisms (Akhavan *et al.*, 2014).

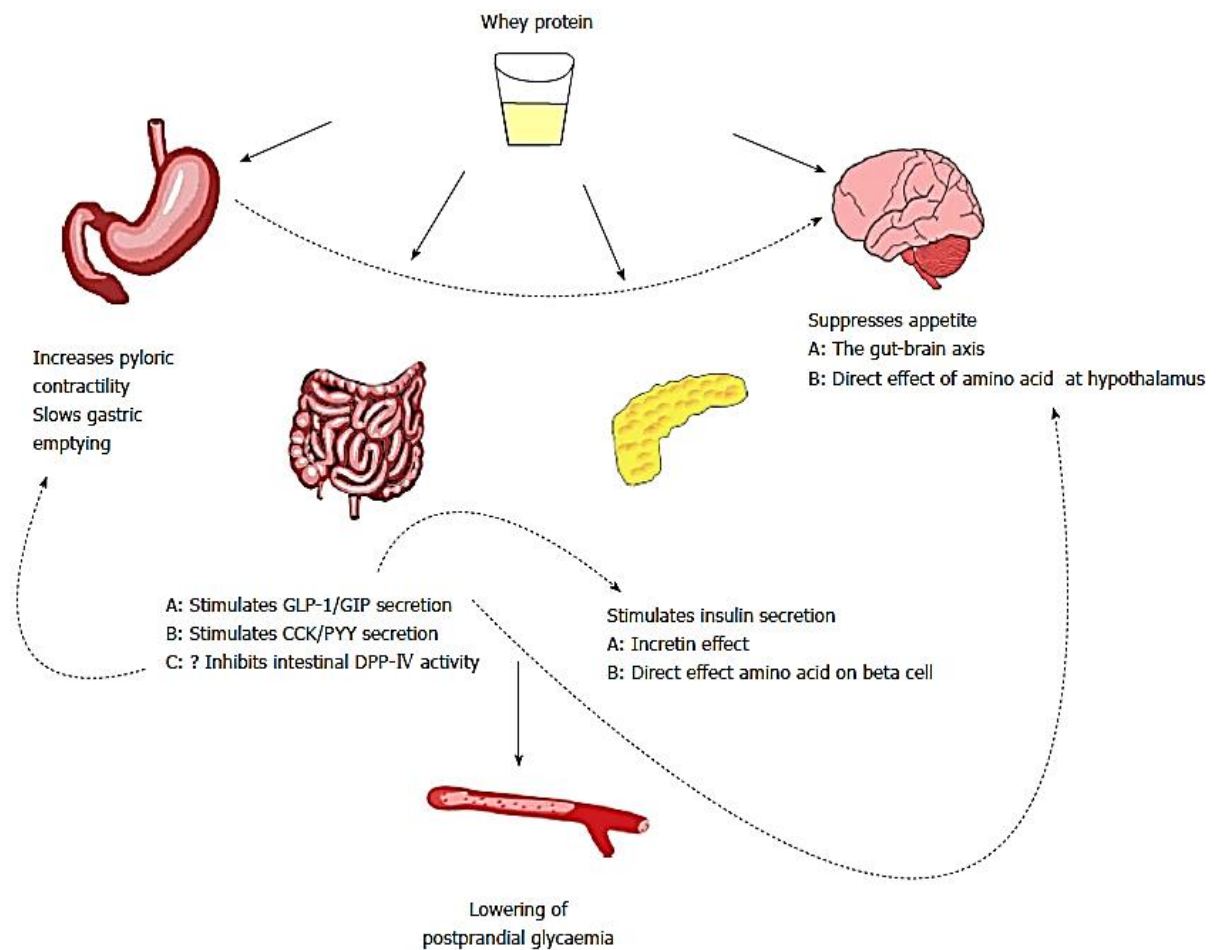


Figure 2.2 Putative mechanisms by which whey protein may influence postprandial glycaemia. Adapted from Mignone *et al.* (2015). GLP-1, glucagon-like peptide 1; GIP, glucose-dependent insulinotropic polypeptide; CCK, cholecystokinin; PYY, peptide YY; DPP-IV, dipeptidyl peptidase-IV.

Although not fully understood, evidence suggests that whey protein enhances postprandial insulinaemia via direct and indirect pathways. It is well established that protein, or more specifically amino acids, stimulate insulin secretion (Floyd *et al.*, 1966), an effect that is present in both healthy individuals (Nilsson *et al.*, 2004) and those with T2DM (van Loon *et al.*, 2003). Whey protein is a rich source of EAAs, known to be potent insulin secretagogues (Floyd *et al.*, 1970). This is particularly the case for the BCAAs leucine, isoleucine and valine, of which whey protein has a high content (Ha & Zemel, 2003). Leucine is of particular

interest in this regard, as it can directly stimulate insulin secretion from pancreatic β -cells via inhibition of ATP-sensitive potassium channels, leading to depolarisation of the β -cell membrane and subsequent insulin secretion (Gao *et al.*, 2003). When intact whey protein or a mixture of amino acids including BCAAs were administered to healthy individuals (combined with 25 g glucose), both drinks raised insulin concentrations similarly, suggesting that the action of whey on postprandial glycaemia is largely associated with its BCAA content (Nilsson *et al.*, 2007). However, the GIP response was 80% greater following intact whey compared to glucose control, whereas it was not different for the amino acid mixtures. This is indicative of bioactive peptides present in intact whey, or formed during digestion, influencing the incretin response. Indeed, whey also stimulates GLP-1 release in healthy males (Akhavan *et al.*, 2014). Both hormones have strong insulinitrophic effects (Adams & Broughton, 2016), therefore whey stimulated incretin release may be responsible for an indirect influence on the insulin response, an effect observed *in vitro* (Salehi *et al.*, 2012).

The efficacy of the incretin hormone response is diminished by rapid degradation to inactive forms by the dipeptyl peptidase-IV (DPP-IV) enzyme (Power *et al.*, 2014). Dairy proteins, including whey, have emerged as potential endogenous inhibitors of DPP-IV, however evidence in humans is limited. Administration of whey has been associated with a significant reduction in DPP-IV activity in mice (Gunnarsson *et al.*, 2006), while a peptide identified from whey β -lactoglobulin hydrolysate is associated with moderate DPP-IV inhibition *in vitro* (Tulipano *et al.*, 2011). In humans with T2DM, consuming 50 g whey protein compared with water prior to a high GI breakfast reduced postprandial glycaemia while stimulating insulin and GLP-1 responses, however no differences in DPP-IV activity were detected (Jakubowicz *et al.*, 2014). One explanation may be that DPP-IV inhibition in the small

intestine may not have been detectable in the plasma, with the possibility that this is another indirect mechanism where whey exerts an effect on insulin secretion.

The most likely mediator of insulin-independent actions of whey on glycaemia is a result of modulation of gastric emptying, since slowing of gastric emptying can diminish postprandial glucose excursions (Horowitz *et al.*, 1993). The coagulation of casein in the stomach is partly responsible for its effect of slowing gastric emptying (Adams & Broughton, 2016), however Calbet and Holst (2004) observed similar rates of gastric emptying following whey and casein ingestion. The addition of whey protein to a carbohydrate load reduces postprandial glucose while displaying delayed gastric emptying (Hoefle *et al.*, 2015), and addition of whey to a preload has been reported to slow gastric emptying of a subsequent meal in healthy (Akhavan *et al.*, 2010) and diabetic (Ma *et al.*, 2009) populations. The gastric emptying and incretin related properties of whey consumption in the context of attenuating postprandial glucose excursions appear to be interdependent, as GLP-1 also slows gastric emptying and suppresses glucagon secretion (Mignone *et al.*, 2015).

The effects of whey protein on satiety or subsequent food intake are thought to be mediated predominantly by gut hormones, specifically via stimulation of GLP-1, CCK and PYY, in addition to suppression of ghrelin, however findings are currently inconsistent. Commercial whey products contain 15-20% caseinomacropeptide which is associated with CCK release (Anderson *et al.*, 2004), and CCK has been reported to increase following whey protein consumption in some (Bowen *et al.*, 2007; Bowen *et al.*, 2006b), but not all (Akhavan *et al.*, 2014) studies. Bowen *et al.* (2006a) reported prolonged suppression of ghrelin as well as

elevated GLP-1 and CCK following whey, soy and gluten preloads compared to glucose, which resulted in a significant reduction in subsequent food intake.

A direct effect of elevated plasma amino acids may also be involved in enhancing satiety with whey ingestion. Intracerebroventricular injection of leucine has been documented to suppress food intake in rats (Morrison *et al.*, 2007), indicating a potential central effect of whey protein on appetite. There is also evidence that whey influences the other side of the energy balance equation through an increase in diet-induced thermogenesis. Acheson *et al.* (2011) observed that whey produced favourable effects on postprandial energy expenditure compared to either casein or soy when contributing 50% of meal energy. Hursel *et al.* (2010) reported greater thermogenesis following a high protein yoghurt (41% of energy) with additional whey protein versus an energy-matched normal protein yoghurt (15% of energy). In addition to directly affecting energy balance, energy expenditure from thermogenesis has been proposed to induce satiety (Westerterp-Plantenga *et al.*, 1999).

The mechanisms explaining the effect of whey protein on postprandial lipaemia remain unclear, however it has been suggested that the reduction in circulating triglyceride-rich chylomicrons following whey supplementation may be related to the relatively fast rates of digestion and absorption of whey and subsequent appearance of BCAA in the plasma (Pal *et al.*, 2010b). It has also been speculated that whey may lead to reduced production of chylomicrons in addition to greater clearance through stimulation of LPL by insulin (Mortensen *et al.*, 2009), however further work is required here.

2.5.2.2.5 Limitations of the current evidence

Currently, many studies have utilised high GI, carbohydrate-rich test meals such as powdered potato with (Ma *et al.*, 2009) or without (Frid *et al.*, 2005) additional glucose, or have mixed glucose into a whey protein beverage (Gunnerud *et al.*, 2013; Nilsson *et al.*, 2007; Petersen *et al.*, 2009) served as a test breakfast. This enables assessment of the efficacy of whey to attenuate greatly elevated postprandial glycaemia, however it may be valuable to investigate the response following consumption of whey with realistic breakfast-type foods. The consumption of test beverages in place of solid foods may also have implications when assessing appetite, since satiation is initially affected by sensory features (Benelam, 2009).

There is a dearth of studies investigating the pure addition of whey to a meal as a supplemental strategy. A number of well controlled studies have matched comparison treatments for energy (Bowen *et al.*, 2006a; Chungchunlam *et al.*, 2015) or macronutrient content (Gunnerud *et al.*, 2012a; Mortensen *et al.*, 2012), however adherence in a naturalistic setting may be easier when adding protein before or alongside a meal, rather than substituting it for other nutrients in that meal. Similar designs have supplemented whey by adding it to glucose (Petersen *et al.*, 2009; Zafar *et al.*, 2013) or maltodextrin (Hoefle *et al.*, 2015), but examples of addition to mixed-macronutrient meals are lacking.

The balance between providing an efficacious dose to reduce postprandial hyperglycaemia, ensuring palatability, and avoiding overconsumption of excess energy, should be carefully considered when designing studies. Studies in overweight/obese non-diabetic populations have all supplemented 45-55 g protein. A 45 g whey protein preload, as administered by Pal

et al. (2010b), contains ~180 kcal which, if consumed regularly, may have a detrimental impact on energy balance.

To the author's knowledge, only one study (Ma *et al.*, 2009) has investigated the effect of timing of whey protein before or alongside a meal on subsequent metabolic responses. Several studies have administered whey at a set time before a standardised or *ad libitum* meal under all conditions, while fewer studies have administered whey protein alongside or immediately prior to a test meal. The large variation in preload timings (15-180 minutes) makes direct comparison of the most efficacious supplementation strategy problematic. Furthermore, no studies have investigated whether consumption of whey following a meal, would be beneficial. In addition, there are currently no studies that have investigated the effects of whey protein consumption on postprandial responses beyond a single isolated meal. Frid *et al.* (2005) examined post breakfast and post lunch responses, however whey was given with both meals meaning that interpretation of any second meal effects of prior whey consumption is confounded by subsequent whey ingestion. Given that a high (non-dairy) protein breakfast has been reported to attenuate postprandial glucose excursions following a standard lunch meal in participants with T2DM (Park *et al.*, 2015), this may be novel area to investigate.

An area of great interest in recent years has been the consumption of whey protein following resistance exercise, due to its stimulatory effect on muscle protein synthesis (Witard *et al.*, 2014). However, an area of great paucity in the literature concerns the influence of whey protein supplementation following aerobic exercise, particularly regarding subsequent postprandial responses. Studies have investigated the effects milk (Rumbold *et al.*, 2015) or

whey protein (Clayton *et al.*, 2014) consumption after moderate-intensity cycling exercise on food intake responses 60 minutes later, reporting reduced *ad libitum* energy intake compared to an energy-matched carbohydrate drink (Rumbold *et al.*, 2015) or low energy placebo (Clayton *et al.*, 2014). However, considering that a single bout of exercise produces divergent responses in subsequent glucose tolerance (see section 2.2.4), the impact of post exercise whey consumption on postprandial metabolic responses warrants attention, particularly in sedentary individuals.

2.6 Summary

A large body of evidence has associated consumption of dairy products, or more specifically dairy proteins, with reduced prevalence of obesity, metabolic syndrome and T2DM. Furthermore, a growing number of studies provides acute evidence of favourable metabolic and appetite-related effects of whey protein consumption at breakfast, which may be responsible for driving this association. Despite some inconsistencies, the majority of evidence suggests a beneficial effect of consuming whey protein before or with a meal on the subsequent glycaemic response in healthy and diabetic populations. These effects are indicative of the potential for whey protein supplementation to be effective in the prevention or management of declining metabolic health in healthy individuals, and those at risk of developing metabolic disorders.

Previous studies show considerable variation regarding the timing and dosing of supplemental whey, in addition to the method of co-ingestion with other macronutrients, while it is not known whether the acute beneficial effects extend beyond breakfast to a

subsequent meal. Furthermore, the ability of supplemental whey protein to augment the postprandial milieu following prior aerobic exercise is unclear. Therefore, the aim of this thesis is to investigate practical strategies for the efficacious supplementation of whey protein to positively augment postprandial meal handling in normal-weight and obese populations.

CHAPTER 3

General Methods

Methods that were repeated across multiple chapters within this thesis are described below. Ethical approval for all studies was granted by the Faculty of Health and Life Sciences Research Ethics Committee at Northumbria University.

3.1 Participants

Participants were recruited from the local community in addition to the student and staff population at Northumbria University, Newcastle Upon Tyne, UK. Methods of recruitment included email, poster advertisement and oral presentation. Individuals who responded to initial recruitment calls were sent written information in the form of a participant information sheet (see Appendix A for an example participant information sheet), detailing the nature of the study and comprehensive details of the time commitment and demands involved, as well as any potential risks and benefits of participation.

Prospective volunteers who registered an interest were subsequently screened against the relevant inclusion/exclusion criteria (Table 3.1). During this screening visit a full verbal explanation of the study was given, and volunteers completed an informed consent form (Appendix B) and a health questionnaire (see Appendix C for example health questionnaire). Anthropometric measurements were taken as described in section 3.6.1.

Table 3.1 Inclusion and exclusion criteria for participants across all experimental chapters.

	Chapters 4a and 4b	Chapters 5 and 6
Inclusion	Male	Male
	Aged 18-40 years	Aged 18-55 years
	Regularly physically active	Low level of physical activity
	BMI: 20-30 kg·m ⁻²	BMI > 30 kg·m ⁻²
		Waist circumference > 102 cm
Exclusion	Taking medication that may influence metabolism or digestion	
	Recent or current smoker	
	Cardiovascular, metabolic or renal disease	
	Current illness	
	Regular breakfast skipper	
	Known food allergies or intolerances	
	Eating disorder	
	Inadequate venous access	

In Chapters 4a and 4b, young males who were habitually physically active were recruited, defined as those who regularly completed at least 30 minutes of structured exercise 5 times per week, assessed by self-report. In Chapters 5 and 6, centrally-obese males who were not regularly physically active but otherwise considered healthy (i.e. no reported cardiovascular or metabolic conditions) were recruited. Central obesity is considered an important aspect in the pathogenesis of metabolic syndrome (O'Neill & O'Driscoll, 2015), and is more strongly associated with increased risk of insulin resistance, metabolic syndrome and CVD than BMI alone (Nedungadi & Clegg, 2009). Participants were therefore screened for elevated waist circumference using the WHO threshold associated with the greatest risk of metabolic complications in males (Alberti *et al.*, 2009). For these studies, prospective participants completed the International Physical Activity Questionnaire (Appendix D) (Hagströmer *et*

al., 2006) during screening, and were deemed to meet the inclusion criteria if they were considered to have a “low” physical activity level after analysis using the categorical scoring method. Participants were considered to be ‘breakfast skippers’ if they self-reported as consuming breakfast on two or less occasions in the previous 7 days, and considered themselves as someone who was not a regular breakfast eater (Reeves *et al.*, 2015).

The sample sizes recruited within each study were calculated using data for a difference in the primary outcome of glucose AUC, and its typical error variance, from relevant previous studies. Based on the reduction in glucose AUC observed in normal-weight participants following addition of whey to a standard meal (Gunnerud *et al.*, 2012b), a sample size of nine was deemed sufficient to provide statistical power above 80% with an alpha level of 0.05. When taking dropouts into account, the recruitment of 11 participants was targeted in Chapter 4. Based on the findings of a 16% reduction in glucose AUC following consumption of whey protein with a mixed-macronutrient breakfast in an overweight/obese population (Pal *et al.*, 2010b), it was calculated that a sample size of 11 would provide statistical power above 80%, with an alpha level of 0.05. When taking dropouts into account, a total of 13 participants was targeted in Chapters 5 and 6.

3.2 Experimental design

A single-blind crossover design was implemented in all studies presented within this thesis, whereby participants completed all experimental arms in a randomised, counterbalanced order. An online randomisation tool for researchers (<http://www.randomization.com/>) was used to create a set of balanced treatment order regimens by inputting the number of

experimental arms and expected sample size. Participant identification numbers were then randomly assigned to a treatment schedule using the '=RAND()' function in Microsoft Excel to determine the order of trial completion.

Studies were designed to assess the effects of whey protein supplementation and/or exercise manipulation at breakfast time on acute and second meal postprandial metabolic and appetite responses. Prior to all experimental visits, diet and physical activity was standardised as described in section 3.3. All trials were conducted within the clinical research laboratories at Northumbria University, commencing at the same time (~08:00 am) where possible and separated by at least 3 (Chapters 4 and 5) or 7 (Chapter 6) days.

In Chapter 4 participants completed five trials, comprising four experimental conditions and one control condition. To present appropriate comparisons between conditions, data from this study are presented in two sub-chapters. Chapter 4a describes data collected in trials where a carbohydrate-rich breakfast was consumed alongside the control (no breakfast) trial. Chapter 4b presents data from trials which involved ingesting a fat-rich breakfast, alongside the same control dataset.

3.3 Pre- and post-trial diet and physical activity standardisation

Prior to each experimental visit participants were provided with verbal and written instructions regarding diet and physical activity control measures. For 24 hours prior to arrival participants were instructed to avoid caffeine and alcohol consumption as well as strenuous physical activity. A mixed-macronutrient meal was provided before each visit in

all studies (Table 3.2), and participants were instructed to consume this as their evening meal, approximately 12 hours prior to their arrival time, in order to standardise macronutrient and energy intake as far as possible. Following ingestion of this meal participants were instructed to consume only water (for the remainder of the evening and the following morning), ensuring that they arrived at the laboratory having fasted for approximately 12 hours. In addition, participants were asked to travel to the lab using automated transport where possible, to avoid excessive pre-trial exertion.

Table 3.2 Composition of pre-trial evening meals.

Chapter	Macronutrient Composition (% energy)			Energy (kJ)	Ingredients and Manufacturer
	CHO	FAT	PRO		
4a, 4b	44.2	33.7	20.8	3171	Pasta Bolognese (Heinz, USA), Chocolate pot dessert (Cadbury, UK).
5, 6	37.3	43.7	18.9	3501	Beef Lasagne (Tesco Stores Ltd, UK), Nature Valley oats and honey bar (General Mills, USA).

In Chapters 4a and 4b participants were provided with a self-reported estimated food diary (Appendix E) during their first visit and asked to record all food and beverage intake for 18 hours after leaving the laboratory, concomitant to the recording of interstitial glucose concentrations. Dietary intake during this period was subsequently replicated after leaving the laboratory following the other experimental visits.

3.4 Preparation of test meals

A standardised breakfast meal of porridge oats was consumed in Chapters 5 and 6 which was designed to represent a frequently consumed meal amongst breakfast eaters in the UK (Reeves *et al.*, 2013), providing 1960 kJ of energy (70% carbohydrate, 17% fat, 13% protein) with a GI of 63. To prepare each meal, 54 g rolled porridge oats (Quaker, UK) combined with 260 ml semi-skimmed milk (Tesco Stores Ltd, UK) was cooked in a microwave (900 W) for 130 seconds. After heating, 42.5 g clear honey (Tesco Stores Ltd, UK) was stirred into the semi-liquid porridge mixture which was served to the participant alongside 250 ml of drinking water. Participants were encouraged to consume this meal within 10 minutes.

A standardised lunch meal was administered to test for second meal effects of breakfast interventions in Chapters 4 and 5. This pasta-based meal provided 3446 kJ of energy (50% carbohydrate, 36% fat, 14% protein) with a GI of 54. A 125 g portion of dried fusilli pasta (Tesco Stores Ltd, UK) was combined with 1000 ml water and heated in a microwave (900 W) for 13 minutes, then drained and mixed with 170 g of a tomato-based sauce (Dolmio Tomato and Basil Pasta Sauce, Mars, USA). Grated cheddar cheese (40 g) (Tesco Stores Ltd, UK) was added, along with 15 g olive oil (Tesco Stores Ltd, UK), before mixing thoroughly. The resulting homogenous meal was heated for a further 2 minutes before being served to the participant alongside 350 ml of drinking water. Participants were again instructed to finish the entire meal within 10 minutes. To assess energy intake in the final study of this thesis (Chapter 6), a pasta meal of similar macronutrient composition was served *ad libitum*.

Information regarding the preparation and composition of this meal is presented in section 6.2.5.

The GI of test meals was calculated using the method of Wolever and Jenkins (1986) for calculation of GI for mixed meals, after calculation of the GI contribution of each meal component using published tables (Atkinson *et al.*, 2008).

3.5 Whey protein isolate powder

In all studies, whey protein isolate powder was combined with water to provide a supplemental beverage of the desired protein content. This was provided by Arla Foods Ingredients Group, Viby, Denmark (Chapters 4a and 4b: PSNU27600; Chapters 5 and 6: Lacprodan SP-9225 Instant). Both batches of product were provided as a ready-to-use dry mix powder which was soluble in water and provided a neutral taste, however all beverages were flavoured using energy-free flavouring (FlavDrops, Myprotein, UK) in an attempt to blind participants to the treatment (flavour-matched water was served in placebo trials). Details regarding the specific doses of supplement administered during each study are presented within Chapters 4-6. Full chemical specifications and amino acid profiles of these supplements are provided in Appendix F.

3.6 Data Collection

3.6.1 Anthropometry

Stature was measured to the nearest 0.1 cm using a fixed stadiometer (602VR, Holtain Ltd, UK), and body mass was determined to the nearest 0.05 kg using a digital scale (Seca 704, Seca Ltd, Germany), after ensuring that participants had removed footwear and were wearing lightweight clothing. These values were used to determine BMI by dividing body mass in kilograms by the square of stature in metres ($\text{kg}\cdot\text{m}^{-2}$). In Chapters 5 and 6 waist and hip circumference were measured to the nearest 0.1 cm using a steel anthropometric tape (Lufkin W606PM, Lufkin, USA) according to International Society for the Advancement of Kinanthropometry (ISAK) guidelines (Stewart *et al.*, 2011). The waist measurement was taken at the narrowest point between the lower costal border and the iliac crest, or the mid-point between these landmarks if no obvious narrowing was apparent. Hip circumference was recorded over light clothing at the level of the greatest posterior protuberance. Waist-hip ratio was calculated by dividing waist circumference by hip circumference.

3.6.2 Subjective appetite ratings

Subjective ratings of appetite were captured at baseline and periodically throughout all studies using VAS (Appendix G). The reproducibility of within-subject responses and the sensitivity to experimental manipulations for this technique have previously been established (Flint *et al.*, 2000; Stubbs *et al.*, 2000). Paper-based 100 mm scales were used to record perceptions of hunger, fullness, satisfaction and prospective food consumption (PFC). Each

scale was anchored with statements representing the theoretical minimum and maximum values on the continuum for that aspect of subjective appetite. For example, in order to capture sensations of hunger, participants were asked the question “how hungry do you feel” and were presented with a scale anchored at either end with the statements “I am not hungry at all” and “I have never been more hungry”. Participants marked a vertical line on each scale indicating their feelings at that time point, and were blinded to their ratings at previous time points. The distance in mm between the extreme left of the scale and this mark was measured to provide a quantitative estimate of appetite sensations. Scales were measured independently by two researchers with the mean value recorded.

3.6.3 Blood sampling

Approximately 10 minutes after arrival, a cannula (Chapters 4a and 4b: Venflon 20G, Becton Dickinson, USA; Chapters 5 and 6: Vasofix 22G, B.Braun Melsungen AG, Germany) was inserted into a vein in the antecubital fossa while participants remained in a semi-supine position. A 10 cm connecting tube (Connecta, Becton Dickinson, USA) with three-way tap was attached to the cannula in Chapters 4a and 4b (Figure 3.1a), which was kept patent by flushing with 0.9% (w/v) sodium chloride solution (Posiflush, Becton Dickinson, USA). To ensure that samples were not contaminated by saline the first ~2.5 ml of blood was discarded at each sample point. In Chapters 5 and 6 sterile stylets (22G, B.Braun Melsungen AG, Germany) were inserted to keep the cannula patent between blood samples (Figure 3.1b).

To control for any postural changes in plasma volume participants were instructed to remain in a seated position throughout the protocol where possible. Care was taken to ensure that

participants remained in this position 5 minutes prior to, and during, each blood sample, except for the sample which immediately followed 30 minutes of treadmill walking in Chapter 6. It was assumed that plasma volume would not be influenced significantly by the low-moderate intensity exercise prescribed in Chapter 6 ($\sim 50\% \dot{V}O_{2\text{peak}}$), as studies have indicated that plasma volume does not significantly change when exercise bouts of greater intensity and duration were employed (Broom *et al.*, 2007; Burns *et al.*, 2007). Concentrations of all analytes in plasma are therefore presented as uncorrected values.

At each sample point, 10 ml of whole blood was drawn into a syringe from the cannula (Figure 3.1c). A 20 μl sodium heparinised capillary tube was filled with whole blood in Chapter 4 for immediate determination of glucose concentration. The remaining blood was transferred into a 10 ml Ethylenediaminetetraacetic acid (EDTA) coated tube (Vacutainer, Becton Dickinson, USA) and immediately centrifuged at 1734 *g* and 4°C for 10 minutes (Allegra X-22R, Beckman Coulter, USA). Plasma was aliquoted into separate microtubes and stored at -80°C for subsequent analysis.

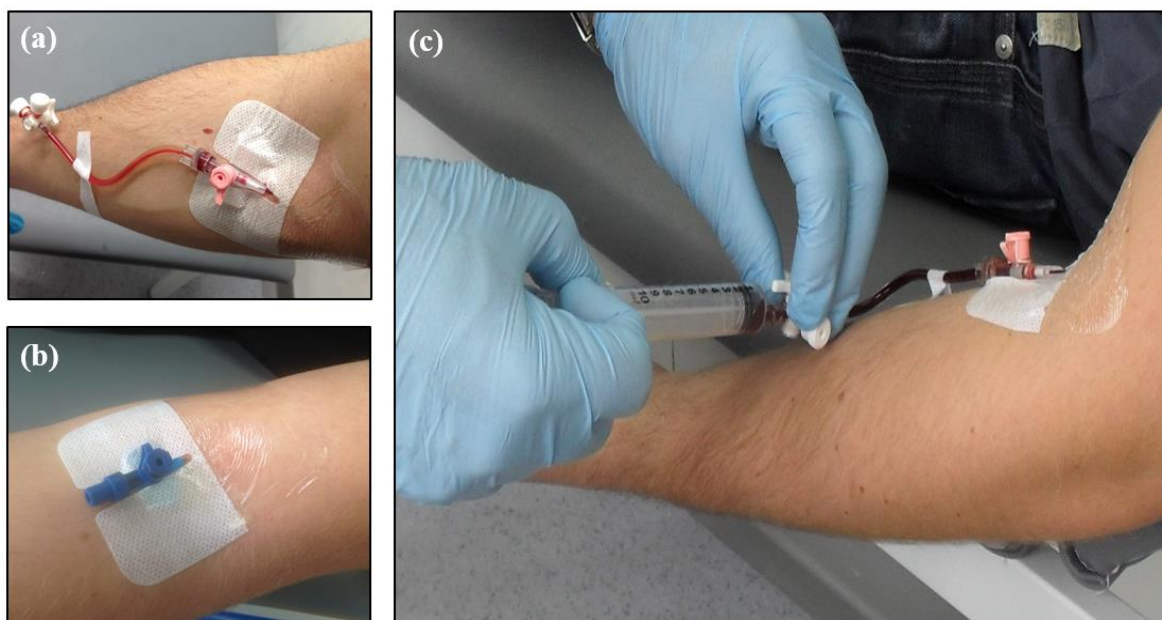


Figure 3.1 Images of venous blood sampling equipment used in Chapters 4a and 4b (a) and Chapters 5 and 6 (b). Blood was drawn into a 10 ml syringe at each sample point (c).

In Chapters 5 and 6, capillary blood samples (20 μ l) were collected immediately following each venous blood draw using the finger-prick technique. Additional samples were collected at 5 and 10 minutes post meal in order to increase the resolution of the blood glucose curve during the period where rapid changes may occur. After cleaning the fingertip, a single-use lancet (Accu-Chek Safe-T Pro Plus, Roche, Switzerland) was used to prick the skin and induce bleeding (Figure 3.2a). After wiping the first drop of blood with a tissue, the sample was drawn into a 20 μ l sodium heparinised capillary tube (EKF Diagnostics, UK) (Figure 3.2b) which was placed in haemolysing solution (Figure 3.2c) for immediate analysis of glucose as described in section 3.6.4.1.

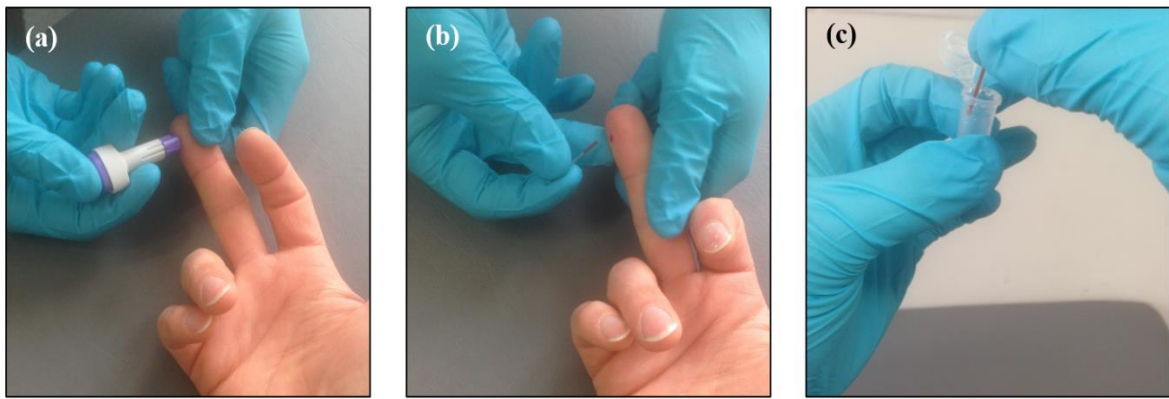


Figure 3.2 Images of capillary blood sampling technique. A single-use lancet is used to prick the fingertip (a), a capillary tube is filled (b) and placed in haemolysing solution (c).

Whilst venous blood is widely used to determine glucose concentration, metabolism at tissues within the forearm characteristically results in lower values than either capillary or arterial blood (Eriksson *et al.*, 1983; Liu *et al.*, 1992), particularly in the post-meal milieu where glucose uptake may be stimulated. Postprandial glycaemia was the primary outcome of interest in the studies presented in this thesis, therefore it was felt that using capillary blood offered a suitable method of sampling which provides values that do not differ markedly from arterial blood (Liu *et al.*, 1992), without the practical constraints associated with collecting arterial blood. This design also allows for simultaneous measurement of venous and capillary blood in Chapters 5 and 6. Whilst there may be absolute differences between glucose values collected from these compartments, it should be noted that the pattern of response is similar, indicating that the use of venous blood glucose in Chapter 4 still provides a valid method of assessing changes of within-subject blood glucose between conditions.

3.6.4 Blood sample analysis

3.6.4.1 Glucose

Capillary tubes filled with whole blood (20 μ l) were placed into microtubes and combined with 1 ml haemolysing solution prior to analysis. Glucose concentrations were determined simultaneously using the Biosen C_line analyser (EKF Diagnostics, UK) (Figure 3.3). The accuracy and precision of this analyser has been previously validated against a reference instrument (Nowotny *et al.*, 2012). This utilises the enzymatic-amperometric method, whereby glucose oxidase catalyses the conversion of glucose to gluconic acid. Hydrogen peroxide is produced which is oxidised at the electrode generating electrons, and the resulting current is proportional to the initial glucose concentration.



Figure 3.3 Biosen C_line analyser for determination of blood glucose concentration.

3.6.4.2 Insulin

Plasma insulin concentration was determined using a solid-phase enzyme-linked immunosorbent assay (ELISA) based on the sandwich principle (IBL International, Hamburg, Germany). All reagents were stored and prepared according to manufacturer instructions. Samples were thawed at room temperature and shaken using a vortex mixer prior to assaying. Aliquots of sample were incubated, along with an anti-insulin antibody conjugated with biotin, in microplate wells coated with monoclonal antibody. After washing off unbound conjugate, a second incubation period was initiated where streptavidin horseradish peroxidase (HRP) enzyme complex binds to the biotin-anti-insulin antibody. After a further washing step, the amount of bound HRP complex is proportional to insulin concentration. A substrate (tetramethylbenzidine) was subsequently added to produce a fluorescent product, the intensity of which was proportional to sample insulin concentration. After incubating for 15 minutes at room temperature this reaction was stopped by adding sulphuric acid. The absorbance at each well was determined using a microtiter plate reader at a wavelength of 450 nm (Anthos 2010, Anthos GmbH, Germany). The sensitivity of the assay according to manufacturer instructions was $1.76 \mu\text{IU}\cdot\text{ml}^{-1}$.

A standard curve was created from the known concentration and mean absorbance values of the duplicate standards using Prism 5 software (Graphpad Software, USA). Sample concentrations in $\mu\text{IU}\cdot\text{ml}^{-1}$ were calculated from sample absorbance values by interpolation from the standard curve, and subsequently converted to SI units ($\text{pmol}\cdot\text{l}^{-1}$) by multiplying by a conversion factor of 6 (Staten *et al.*, 2010). In Chapters 5 and 6 samples were diluted 1:3 using zero standard after initial analysis produced values outside of the assay range.

All samples from a participant were analysed on a single plate where possible in order to minimise inter-assay variation. The majority of samples were assayed in singlet, with at least one sample per trial measured in duplicate. Reference samples were assayed on each plate to calculate inter-assay variation. All standards were assayed in duplicate. The intra- and inter-assay coefficients of variation for all assays conducted were $6.9 \pm 0.9\%$ and $10.8 \pm 0.7\%$ respectively.

3.6.4.3 Enzymatic colourimetric assays

NEFA, glycerol and triglyceride concentrations were determined with an automated analyser (RX Daytona, Randox Laboratories, UK) using enzymatic colorimetric assays (Randox Laboratories, UK) according to manufacturer instructions. The analyser was calibrated daily with ready-to-use calibrators supplied within each kit. Quality control material was assayed on two levels for NEFA and triglyceride (Human Assayed Multi-Sera – Level 2 and Level 3, Randox Laboratories, UK) and on one level for glycerol (Glycerol Control, Randox Laboratories, UK). Assay sensitivity according to manufacturer instructions was $0.072 \text{ mmol}\cdot\text{l}^{-1}$, $14.5 \text{ }\mu\text{mol}\cdot\text{l}^{-1}$ and $0.134 \text{ mmol}\cdot\text{l}^{-1}$ for NEFA, glycerol and triglyceride respectively. Samples were analysed in singlet, and all samples from a participant were analysed on the same run where possible. Analyte concentrations were provided in SI units directly by the analyser software. Intra-assay coefficients of variation for all assays conducted were $<7.2\%$.

3.6.5 Continuous glucose monitoring

In Chapters 4a and 4b, participants were fitted with a continuous glucose monitor (CGM) to assess the impact of each intervention on longer-term glycaemia (i.e. 24 hours). The CGM gives continuous (every 5 minutes) measures of interstitial glucose concentration which allows the capturing of high resolution data and thus information regarding both postprandial excursions and glycaemic variability, which are associated with adverse metabolic effects if elevated above normal levels (Ceriello *et al.*, 2008; Fava, 2008). The CGM device used (G4 Platinum, Dexcom, USA) compares favourably to other systems in terms of accuracy (Kropff *et al.*, 2015), and consists of a small indwelling sensor, a transmitter which attaches to the sensor and a handheld receiver which displays the glucose readings (Figure 3.4). The sensor principally utilises the glucose oxidase method described in section 3.6.4.1 to generate an electrical current proportional to the glucose concentration in the interstitium. The transmitter sends a signal via wireless radio frequency to the receiver which displays and records the concentration. Close agreement between capillary glucose and interstitial concentrations recorded by CGM systems has previously been found throughout the glycaemic range (Caplin *et al.*, 2003; Maia & Araújo, 2007).

The sensor was implanted into the subcutaneous tissue of the anterior-medial aspect of the abdomen using the insertion device provided, and placement was replicated across trials. This was carried out at least 48 hours prior to commencement of the first trial, allowing a period of stabilisation to occur following sensor initiation. The CGM was calibrated by the participant inputting a minimum of four capillary blood glucose readings per day, obtained from a blood glucose meter (Accu-Chek Mobile, Roche, Switzerland) using the finger-prick

technique described in section 3.6.3. As a consequence of the potential for a physiological time delay between blood and interstitial concentrations, participants were instructed to calibrate prior to meals in order to ensure that interstitial fluid glucose was in equilibrium with capillary glucose (Bequette, 2010). Data was captured for 24 hours pre-trial and for 24 hours post-test breakfast, after which the sensor was removed.

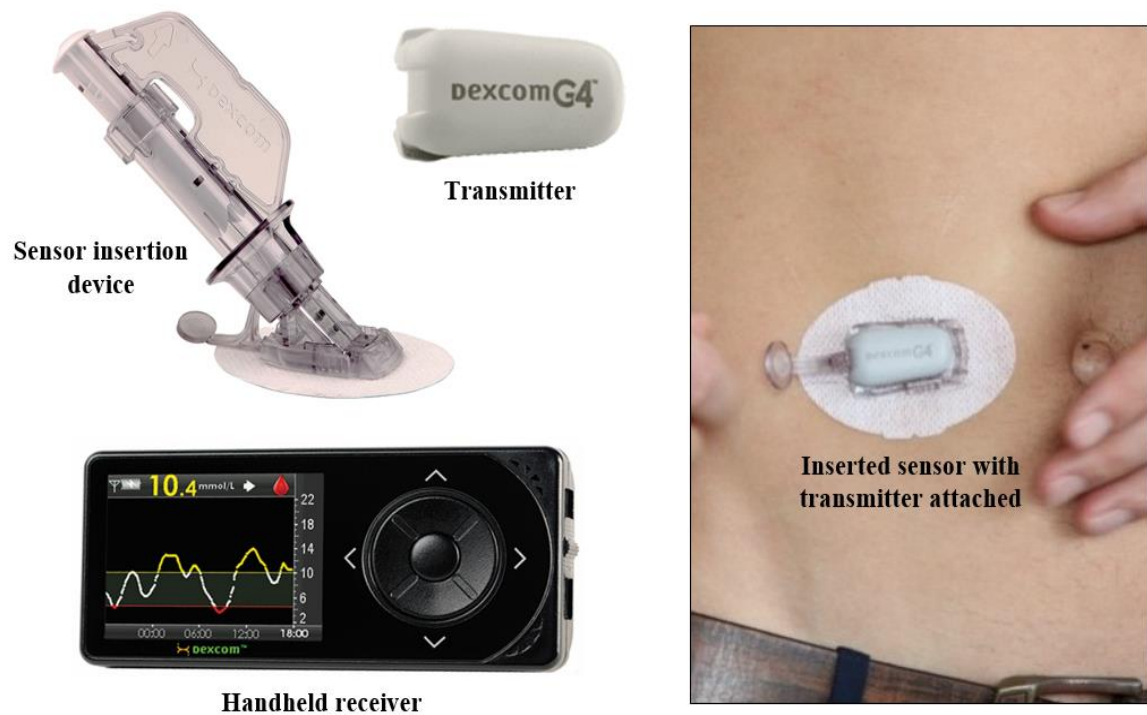


Figure 3.4 Dexcom G4 Platinum continuous glucose monitoring system.

3.7 Data processing

Serial measures of blood analytes, subjective appetite responses and CGM data were used to calculate AUC using the trapezoidal method, which involves addition of the areas under the graph between each pair of consecutive observations. The resultant total AUC includes all area below the response curve in order take account of situations where concentration fell below baseline (Matthews *et al.*, 1990). CGM data were analysed for 24 hours following initiation of breakfast consumption, with a total of 288 sample points recorded during each experimental condition. Data were split into 6 hour epochs for analysis of glycaemic exposure (AUC) and variability. Relative glycaemic variability was expressed as the overall glucose coefficient of variation (%CV), calculated by dividing the standard deviation (SD) with the mean and multiplying by 100. Previous studies have indicated a high degree of correlation between the SD and other parameters of glycaemic variability, such as mean amplitude of glucose excursions (Rodbard, 2009).

Fasting levels of insulin resistance were characterised for participants across all chapters by utilising the homeostatic model assessment of insulin resistance (HOMA-IR) which was calculated using the following equation (Matthews *et al.*, 1985):

$$\text{HOMA-IR} = \frac{\text{Fasting Plasma Glucose (mmol}\cdot\text{l}^{-1}) \times \text{Fasting Plasma Insulin (}\mu\text{IU}\cdot\text{ml}^{-1})}{22.5}$$

Insulin sensitivity was assessed across chapters using the Matsuda Insulin Sensitivity Index (ISI), calculated using fasting and postprandial concentrations of plasma glucose (mg·dl⁻¹) and plasma insulin (μIU·ml⁻¹) using the following equation (Matsuda & DeFronzo, 1999):

$$\text{Matsuda-ISI} = \frac{10000}{\sqrt{(\text{Fasting Glucose} \times \text{Fasting Insulin}) \times (\text{Mean Glucose} \times \text{Mean Insulin})}}$$

In order to combine the four separate aspects of subjective appetite assessment detailed in 3.6.2 into a summary measure of perceived appetite, the equation below was applied to calculate combined appetite score (CAS), as used previously (Gonzalez & Stevenson, 2014):

$$\text{CAS} = \frac{\text{hunger} + \text{prospective consumption} + (100 - \text{fullness}) + (100 - \text{satisfaction})}{4}$$

This composite measure is reported alongside individual appetite ratings in Chapters 4-6.

3.7.1 Statistical analysis

Statistical analysis was conducted using the Statistical Package for the Social Sciences (SPSS version 21, IBM, USA). Matsuda ISI, baseline values and AUC (all variables measured over time) were assessed using one-way repeated measures analysis of variance (ANOVA). Blood and plasma analyte concentrations and subjective appetite responses were tested for differences between conditions over time using two-way (condition x time) repeated measures ANOVA. Mauchly's test was used to check the assumption of sphericity for repeated measures analysis, and the degrees of freedom were adjusted using the appropriate correction if this assumption was violated. Post hoc analysis was performed upon identification of significant main effects and the Bonferroni correction was used to correct

the level of alpha for multiple comparisons. The level of statistical significance was set at $p < 0.05$ and data are presented as mean \pm standard error of the mean (SEM).

CHAPTER 4a

Acute and second-meal metabolic and appetite responses following co-ingestion of whey protein with a carbohydrate-rich breakfast in normal-weight males

4.1.1 Introduction

It is widely acknowledged that obesity predisposes individuals to increased risk of disorders including metabolic syndrome, T2DM and CVD (Wang *et al.*, 2011), characterised by insulin resistance, which is positively correlated with BMI (Shakher & Barnett, 2009). However, postprandial hyperglycaemia, independent of weight status, has an increasingly recognised role in metabolic impairment and contribution to CVD risk (Bianchi *et al.*, 2008; Gerich, 2006; Monnier *et al.*, 2012), even when fasting glucose is in the normal range (Ning *et al.*, 2012). It may therefore be appropriate for interventions aimed at ameliorating postprandial glycaemic excursions, and improving metabolic health in general, to be targeted at the wider population rather than just ‘at risk’ groups, i.e. those with obesity or IFG.

A growing body of evidence implicates manipulation of meal macronutrient content, including protein ingestion, on markers of metabolic health (Leidy *et al.*, 2015b; Mateo-Gallego *et al.*, 2016; Westerterp-Plantenga *et al.*, 2012). Dairy proteins in particular, have been investigated extensively (Bjørnshave & Hermansen, 2014; Pasin & Comerford, 2015), and recent studies point to whey protein as having potentially advantageous effects including reduced postprandial glycaemia (Akhavan *et al.*, 2010; Frid *et al.*, 2005) and lipaemia (Mortensen *et al.*, 2009; Pal *et al.*, 2010b), potentially mediated by an increased insulin response (Gunnerud *et al.*, 2013; Nilsson *et al.*, 2007). Whey proteins promote insulin secretion to a greater extent than other proteins sources in lean individuals (Pal & Ellis, 2010), with a high concentration of BCAAs, particularly leucine, and fast rate of appearance as likely integral mediators (Esteves de Oliveira *et al.*, 2011; Pal & Radavelli-Bagatini, 2013). The subsequent synthesis and secretion of the incretin hormones GIP and GLP-1, due to the presence of amino acids and peptides derived from whey protein digestion (Ricci-Cabello *et al.*, 2012), also likely play an important role in the enhanced insulinaemic response. Incretin hormones increase both fasting and postprandial insulin release (Sousa *et al.*, 2012) and their presence, in concert with increased plasma insulin concentrations, may also lead to appetite suppressive effects (Jakubowicz & Froy, 2013).

Previous studies have investigated the effects of whey protein when consumed prior to, or alongside, a meal on postprandial glycaemia in normal-weight (Gunnerud *et al.*, 2012b; Pal & Ellis, 2010), overweight (Bowen *et al.*, 2007; Pal *et al.*, 2010b) or diabetic populations (Frid *et al.*, 2005; Ma *et al.*, 2009). When whey protein (18.2 g) is ingested at the same time as a high GI lunch meal, a 21% reduction in glucose AUC was reported in participants with T2DM (Frid *et al.*, 2005). A dose-dependent reduction in glucose AUC has also been shown

in healthy individuals (Gunnerud *et al.*, 2013). Studies have regularly prescribed large doses of whey protein which may not be practical to consume alongside a meal and involves intake of relatively high amounts of additional energy. Typically, doses between 45 and 50 g have been employed (Anderson *et al.*, 2004; Bowen *et al.*, 2007; Ma *et al.*, 2009). Furthermore, these studies characteristically involve short observation periods, e.g. 120 minutes (Chungchunlam *et al.*, 2015; Nilsson *et al.*, 2007), and the observation of single meal responses (Hoefle *et al.*, 2015).

The influence on subsequent feeding and metabolism is of importance as the macronutrient composition of a breakfast meal has been demonstrated to influence the metabolic response following subsequent feeding in healthy individuals (Frape *et al.*, 1997) and those with T2DM (Park *et al.*, 2015). It is currently unclear whether the potential beneficial effects of whey protein on the acute postprandial milieu extend beyond a single breakfast meal to have protective effects at lunch or later during the day. Thus, investigating the potential second meal effects of whey protein on glycaemia, insulinaemia, lipaemia and appetite may be important in identifying efficacious strategies to prevent metabolic deterioration in currently healthy individuals, while providing further insights into the supplemental use of whey protein for health. Therefore, the aim of this study was to determine the acute postprandial metabolic and appetite responses in normal-weight individuals following a carbohydrate-rich breakfast, with and without co-ingestion of whey protein, in addition to responses following subsequent feeding.

4.1.2 Methods

4.1.2.1 Participants

Ten normal-weight male participants who were free from metabolic disease (age: 24 ± 1 years; mass: 79.7 ± 1.2 kg; stature: 1.81 ± 0.02 m; BMI: 24.5 ± 0.7 kg·m⁻², HOMA-IR: 2.1 ± 1.1 arbitrary units), were recruited as described in section 3.1, and according to the criteria listed in Table 3.1. All participants provided written informed consent prior to inclusion. This study was registered at clinicaltrials.gov as NCT02414061.

4.1.2.2 Experimental design

A crossover design was implemented with three experimental conditions consisting of a carbohydrate breakfast (CHO), carbohydrate breakfast with supplemental whey protein (CHO+WP) and no breakfast control (NB). Participants attended the laboratory on three separate occasions, separated by at least 72 hours, and underwent all three conditions in a randomised and counterbalanced order (section 3.2). The study involved both a laboratory protocol and a home-based element, where interstitial glucose concentrations were continuously sampled for 18 hours after leaving the laboratory on each occasion. Prior to each visit, physical activity and dietary intake was standardised (section 3.3) and a meal was provided which participants were instructed to consume 12 hours before arrival at the laboratory (Table 3.2).

Upon arrival at ~0800 hours, a cannula was inserted into an antecubital vein and a baseline blood sample collected (see section 3.6.3). Subjective appetite sensations were also sampled at baseline through completion of VAS as described in section 3.6.2. Participants subsequently consumed one of two test breakfasts (CHO or CHO+WP) or ingested a reference bolus of water (NB), with the remainder of the protocol being identical under all conditions (Figure 4.1). Participants remained in a rested state for 180 minutes, after which the same standardised, mixed-macronutrient lunch meal was provided under all conditions. After a further 180 minutes, the cannula was removed and participants could leave the laboratory. Dietary intake for the following 18 hours was replicated on each occasion as described in section 3.3.

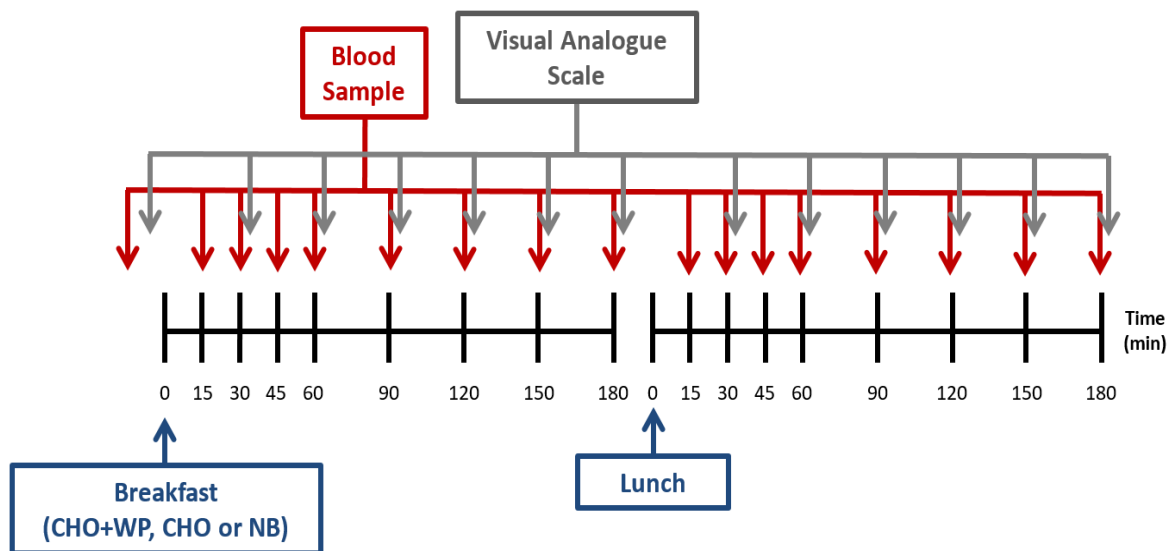


Figure 4.1 Schematic layout of laboratory experimental protocol. CHO, high carbohydrate breakfast trial; CHO+WP, high-carbohydrate breakfast with added whey protein trial; NB, no breakfast trial.

4.1.2.3 Test meals

The energy content and macronutrient composition of the test meals provided in each condition are displayed in Table 4.1. The breakfast meal administered in CHO and CHO+WP consisted of white wheat bread (95 g) and strawberry jam (48 g) with an orange juice beverage (200 ml) which had a GI of 63. Water (250 ml) was provided in an opaque bottle and flavoured with 0.5 ml energy-free vanilla flavouring (FlavDrops, Myprotein, UK) in the CHO trial. This process was repeated for CHO+WP, with the addition of 24 g whey protein isolate powder dissolved in the flavoured water. The whey powder had a protein content of 83% (Appendix F1), providing 20 g protein per serving, which has previously been identified as an efficacious dose for reducing glycaemia (Abou-Samra *et al.*, 2011). These beverages were matched for flavour, however the difference in viscosity due to the presence of the protein was not accounted for. No reference was made to which trial was being conducted throughout, and participants were therefore blinded to the condition in breakfast consumption trials. Further water was provided alongside breakfast, the volume of which was trial-dependent in order to standardise meal volumes across conditions. In the NB trial, an isovolumetric bolus (675 ml) of water was consumed by participants at the applicable time point. Further water was not administered during the postprandial period in order to maintain standardisation and control for any potential effects of gastric volume on appetite suppression (Wang *et al.*, 2008). A timer was started upon initiation of consumption in all trials, and participants were encouraged to consume the meal within 5 minutes. An identical pasta meal was served at lunch in all conditions, which was prepared as described in section 3.4. Water (350 ml) was also served alongside the lunch meal, and was withheld in the post lunch period.

Table 4.1 Energy and macronutrient composition of breakfast and lunch test meals.

		Energy kJ (kcal)	Carbohydrate g (E%)	Fat g (E%)	Protein g (E%)
Breakfast	CHO	1799 (430)	93 (88)	2 (4)	9 (8)
	CHO+WP	2172 (519)	95 (75)	2 (3)	29 (22)
	NB	0 (0)	0 (0)	0 (0)	0 (0)
Lunch		3446 (824)	104 (50)	33 (36)	29 (14)

E%: percentage of total meal energy; CHO: high-carbohydrate breakfast trial; CHO+WP: high-carbohydrate breakfast with added whey protein trial; NB: no breakfast trial.

4.1.2.4 Blood sampling and analysis

Venous blood samples were collected at regular intervals throughout (baseline, 15, 30, 45, 60, 90, 120, 150 and 180 minutes post-breakfast, and corresponding post lunch time points (Figure 4.1)). Samples were collected with subjects in a seated position as described in section 3.6.3, and were analysed for concentrations of blood glucose and plasma insulin, triglyceride, NEFA and glycerol as described in section 3.6.4.

4.1.2.5 Subjective appetite

Subjective appetite ratings were recorded at baseline and at 30 minute intervals following both breakfast and lunch meals using 100 mm paper-based VAS as described in section 3.6.2.

4.1.2.6 Continuous glucose monitoring

Participants were fitted with a CGM (G4 Platinum, Dexcom, USA) at least 48 hours prior to their first trial (see 3.6.5 for detailed information regarding CGM methodology) to allow stabilisation before trial commencement. Interstitial glucose concentrations were continually recorded throughout the laboratory protocol and for a further 18 hours until 24 hours post-breakfast.

4.1.2.7 Statistical analysis

Blood and VAS derived data were analysed over both the post-breakfast (0-180 min) and post-lunch (180-360 min) periods. The time point at 180 minutes post-breakfast was used as the baseline value for the post-lunch period. Due to its delayed appearance in plasma, triglyceride data were also assessed over the full 360 minute laboratory protocol. Total AUC values were calculated using the trapezoidal method and CGM data were processed as described in section 3.7. Statistical analysis was conducted as described in section 3.7.1.

4.1.3 Results

4.1.3.1 Post-breakfast responses

There were no differences between trials at baseline for all measures ($p > 0.05$). Blood glucose concentrations displayed a significant condition x time interaction ($p < 0.001$) and time effect ($p < 0.001$) following breakfast consumption. Postprandial concentrations were

increased in CHO+WP ($p = 0.006$) and CHO ($p = 0.011$) compared to NB at 15 minutes, and remained elevated above NB in the CHO condition at 30 minutes post-breakfast ($p = 0.029$; Figure 4.2a). Comparison of postprandial peak glucose in the breakfast trials revealed a trend for a reduction following the addition of whey protein, however this did not reach significance (CHO+WP: 5.5 ± 0.2 , CHO: 6.4 ± 0.3 mmol·l⁻¹; $p = 0.059$). Blood glucose concentrations were not different between conditions at 45-180 minutes post-breakfast ($p > 0.05$). Furthermore, there were no differences in glucose AUC between conditions across the postprandial period ($p = 0.247$; Table 4.2).

Post breakfast plasma insulin concentrations are displayed in Figure 4.2b. There was a significant condition x time interaction ($p < 0.001$), condition effect ($p < 0.001$) and time effect ($p < 0.001$). Concentrations peaked 30 minutes after breakfast consumption under CHO+WP and CHO, with both similarly elevated above NB at 15-120 minutes post-breakfast, before returning to baseline levels. Insulinaemia across this period was increased by 24.8% with the addition of whey protein to breakfast (CHO+WP vs CHO, $p = 0.033$; Table 4.2), and was greater under both breakfast trials than NB (both $p < 0.001$). Insulin sensitivity during the post-breakfast period, as assessed by Matsuda-ISI, did not differ between breakfast trials (CHO+WP: 10 ± 2 , CHO: 10 ± 2 arbitrary units; $p = 0.656$).

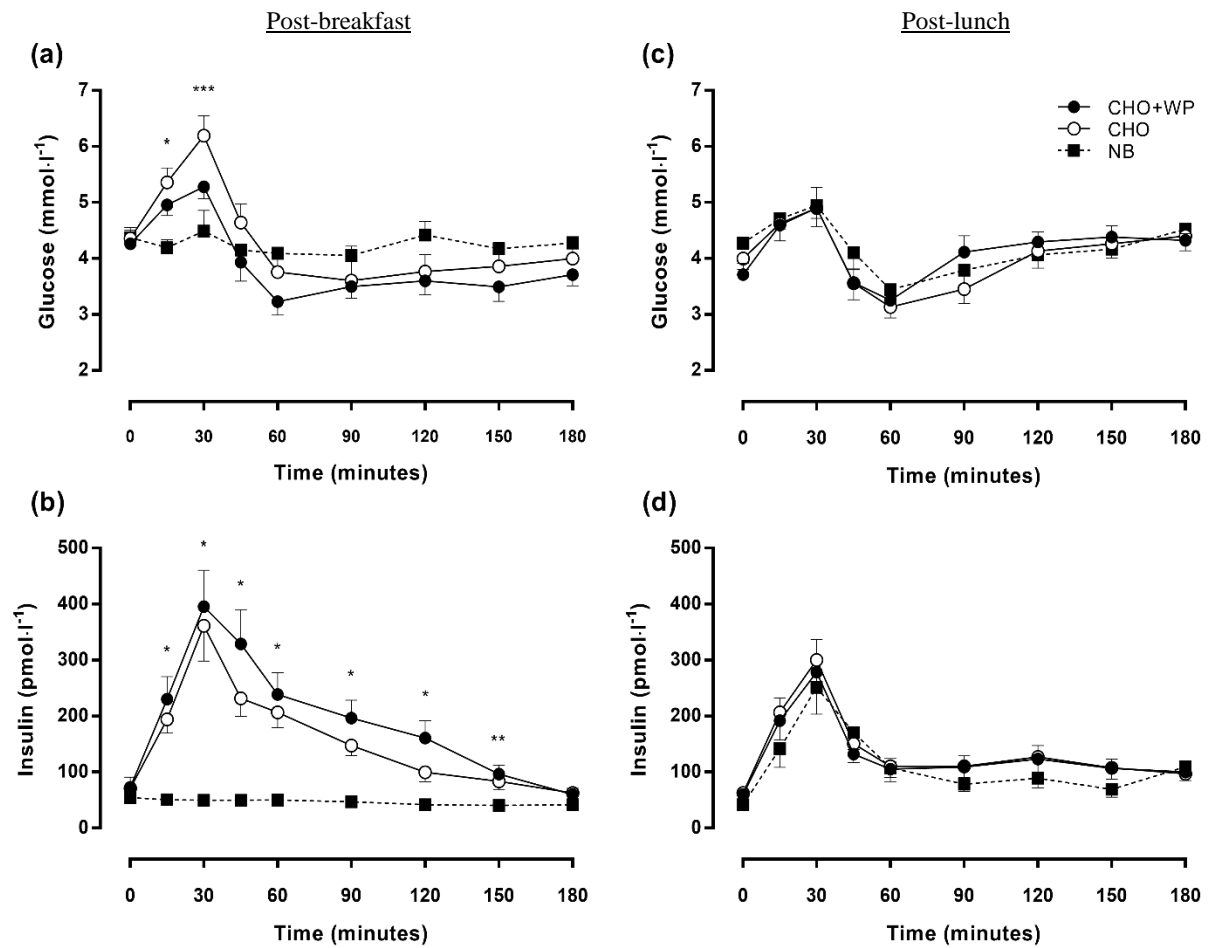


Figure 4.2 Mean \pm SEM temporal changes in blood glucose (a) and plasma insulin (b) during the post-breakfast period (0-180 minutes), and blood glucose (c) and plasma insulin (d) during the post-lunch period (180-360 minutes). Significant differences ($p < 0.05$) between trials at individual time points are defined as follows; *NB vs CHO+WP and CHO, ** NB vs CHO+WP, ***NB vs CHO. CHO+WP: high-carbohydrate breakfast with added whey protein trial; CHO: high-carbohydrate breakfast trial; NB: no breakfast trial.

Table 4.2 Area under the curve for blood and plasma variables during post-breakfast (0-180 minutes) and post-lunch (180-360 minutes) postprandial periods.

		Area under the curve		
		CHO+WP	CHO	NB
Glucose (mmol·min ⁻¹ ·l ⁻¹)	Post-breakfast	690.5 ± 27.6	756.8 ± 42.0	760.6 ± 25.7
	Post-lunch	745.4 ± 24.2	718 ± 26.8	744.2 ± 28.3
Insulin (pmol·min ⁻¹ ·l ⁻¹)	Post-breakfast	34760 ± 4726 ^a	27849 ± 3333 ^b	8306 ± 1446 ^c
	Post-lunch	23522 ± 3387	24646 ± 2824	19935 ± 3349
NEFA (mmol·min ⁻¹ ·l ⁻¹)	Post-breakfast	22.7 ± 5.4 ^a	24.0 ± 5.3 ^a	79.7 ± 14.1 ^b
	Post-lunch	23.0 ± 2.6 ^a	31.6 ± 4.3 ^{a,b}	37.5 ± 3.9 ^b
Glycerol (μmol·min ⁻¹ ·l ⁻¹)	Post-breakfast	6675 ± 1248	7469 ± 924	10244 ± 1702
	Post-lunch	6659 ± 1143	8048 ± 566	7243 ± 1193
Triglyceride (mmol·min ⁻¹ ·l ⁻¹)	Post-breakfast	137.7 ± 23.3	121.2 ± 13.2	118.8 ± 12.2
	Post-lunch	208.2 ± 29.0 ^a	178.2 ± 19.5 ^a	146.8 ± 17.4 ^b
	Total	345.9 ± 51.7	299.4 ± 32.0	265.6 ± 29.1

Data presented as mean ± SEM. Different superscript letters within a row indicates significant difference between conditions ($p < 0.05$). CHO+WP: high-carbohydrate breakfast with added whey protein trial; CHO: high-carbohydrate breakfast trial; NB: no breakfast trial; NEFA: non-esterified fatty acids.

Plasma concentrations of NEFA and glycerol are presented in Figure 4.3. NEFA concentrations were similarly suppressed under CHO+WP and CHO in comparison to NB at 90-180 minutes post-breakfast ($p < 0.05$), while glycerol was significantly lower following CHO+WP than NB at 90-120 minutes post-breakfast ($p \leq 0.013$). Analysis of AUC across the post-breakfast period indicated that NEFA was lower following both breakfast meals compared to NB ($p \leq 0.019$; Table 4.2) without differing between CHO+WP and CHO, and glycerol responses were not different between conditions ($p = 0.092$).

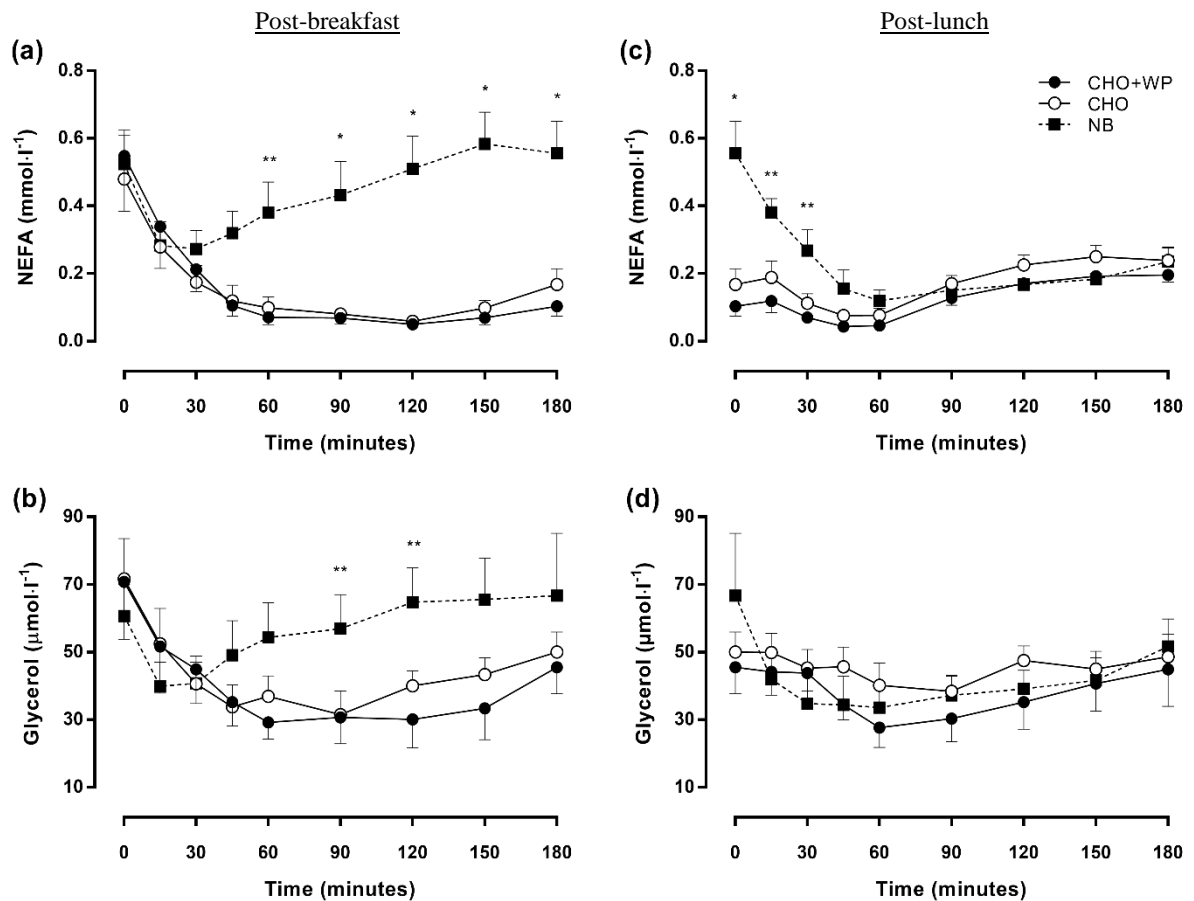


Figure 4.3 Mean \pm SEM temporal changes in NEFA (a) and glycerol (b) during the post-breakfast period (0-180 minutes), and NEFA (c) and glycerol (d) during the post-lunch period (180-360 minutes). Significant differences ($p < 0.05$) between trials at individual time points are defined as follows; *NB vs CHO+WP and CHO, **NB vs CHO+WP. CHO+WP: high-carbohydrate breakfast with added whey protein trial; CHO: high-carbohydrate breakfast trial; NB: no breakfast trial; NEFA: non-esterified fatty acids.

Temporal changes in subjective appetite following breakfast consumption are indicated by time course responses for combined appetite scores (Figure 4.4a). There was a significant condition \times time interaction ($p < 0.001$), condition effect ($p < 0.001$) and time effect ($p < 0.001$) in the post-breakfast period. Appetite was suppressed similarly under CHO+WP and CHO, with combined appetite scores significantly lower than baseline levels at 30 minutes post-breakfast in both conditions ($p \leq 0.001$), whereas appetite was increased above baseline levels from 60 minutes in NB ($p \leq 0.016$). Appetite scores for CHO+WP and CHO

remained below NB from 30-180 minutes post-breakfast ($p < 0.05$). Combined appetite score AUC was greater under NB than CHO+WP and CHO ($p \leq 0.001$; Table 4.3), with similar suppression observed under both breakfast trials ($p = 0.974$). This observation is replicated for the component measures of hunger, fullness, PFC and satisfaction (Table 4.3).

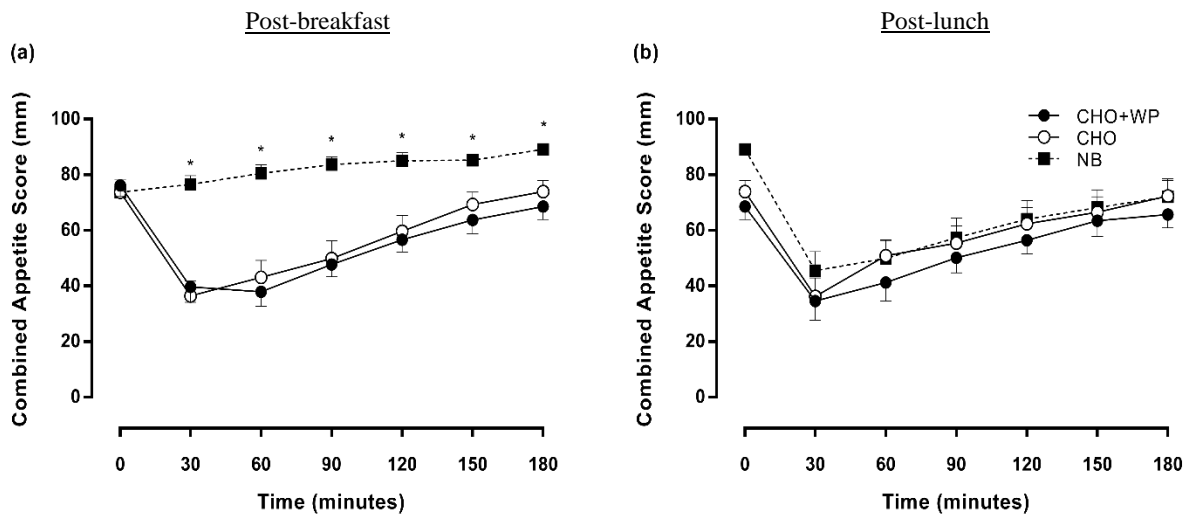


Figure 4.4 Mean \pm SEM temporal changes in combined appetite score during the post-breakfast (a) and post-lunch (b) periods. Significant differences ($p < 0.05$) between trials at individual time points are defined as follows; *NB vs CHO+WP and CHO. CHO+WP: high-carbohydrate breakfast with added whey protein trial; CHO: high-carbohydrate breakfast trial; NB: no breakfast trial.

Table 4.3 Area under the curve for subjective appetite responses during the post-breakfast (0-180 minutes) and post-lunch (180-360 minutes) postprandial periods.

		Area under the curve		
		CHO+WP	CHO	NB
Combined appetite (mm·min ⁻¹)	Post-breakfast	9542 ± 709 ^a	9966 ± 834 ^a	14768 ± 443 ^b
	Post-lunch	9391 ± 866	10338 ± 943	10971 ± 1024
Hunger (mm·min ⁻¹)	Post-breakfast	8756 ± 682 ^a	9442 ± 770 ^a	13406 ± 570 ^b
	Post-lunch	8760 ± 846	9870 ± 898	10307 ± 969
Fullness (mm·min ⁻¹)	Post-breakfast	8571 ± 716 ^a	8291 ± 822 ^a	2853 ± 431 ^b
	Post-lunch	8849 ± 809	7892 ± 981	7061 ± 1033
PFC (mm·min ⁻¹)	Post-breakfast	10463 ± 900 ^a	10880 ± 1082 ^a	15296 ± 435 ^b
	Post-lunch	10224 ± 1140	11151 ± 1028	11780 ± 1143
Satisfaction (mm·min ⁻¹)	Post-breakfast	8479 ± 629 ^a	8168 ± 782 ^a	2775 ± 454 ^b
	Post-lunch	8570 ± 732	7779 ± 912	7139 ± 986

Data presented as mean ± SEM. Different superscript letters within a row indicates significant difference between conditions ($p < 0.05$). CHO+WP: high-carbohydrate breakfast with added whey protein trial; CHO: high-carbohydrate breakfast trial; NB: no breakfast trial; PFC: prospective food consumption.

4.1.3.2 Post-lunch responses

Following lunch insulinaemic and glycaemic responses did not differ between trials (Figure 4.2c-d), with no effect of condition or time x condition interaction on blood glucose or plasma insulin concentrations observed ($p > 0.05$). Postprandial AUC also indicated that glycaemia and insulinaemia (Table 4.2; $p = 0.283$) were not different across the 180 minute period following consumption of a subsequent meal. Post lunch assessments of insulin sensitivity were similar across conditions (CHO+WP: 12 ± 2 , CHO: 11 ± 2 , NB: 16 ± 3 arbitrary units; $p = 0.075$).

A condition x time interaction effect was observed for post-lunch NEFA concentrations ($p < 0.001$). Levels did not significantly differ from pre-lunch concentration in CHO+WP or CHO ($p > 0.05$), but decreased following lunch consumption in NB, with similar concentrations maintained between trials from 45-180 minutes post-lunch ($p > 0.05$; Figure 4.3c). Post-lunch NEFA AUC was reduced in CHO+WP compared with NB ($p = 0.050$), but did not differ significantly from CHO ($p = 0.206$; Table 4.2). There were no differences in plasma glycerol concentrations between trials throughout the post-lunch period ($p > 0.05$; Figure 4.3d).

Deviations in subjective appetite responses between CHO+WP and CHO were not found following lunch ($p > 0.05$; Figure 4.4b). A trend for reduced appetite in CHO+WP compared to NB was detected, however the difference in combined appetite AUC observed was not significant ($p = 0.076$; Table 4.3). This trend may be more closely related to differences in levels of hunger (AUC: CHO+WP vs NB, $p = 0.052$) than other subjective appetite components (all $p \geq 0.099$).

4.1.3.3 Plasma triglyceride

Plasma triglyceride displayed a significant condition x time interaction ($p < 0.001$) over the course of the laboratory protocol. Plasma concentrations did not differ significantly from baseline following breakfast omission ($p > 0.05$), however they were raised above NB in CHO+WP at 270-360 minutes ($p \leq 0.041$) and in CHO at 270-330 minutes post-breakfast (Figure 4.5; $p \leq 0.035$). Triglyceride AUC responses were not different between trials across the full postprandial period ($p > 0.05$), however when analysed over just the post-lunch period

AUC was reduced following prior breakfast omission compared to both CHO+WP ($p = 0.010$) and CHO ($p = 0.028$; Table 4.2).

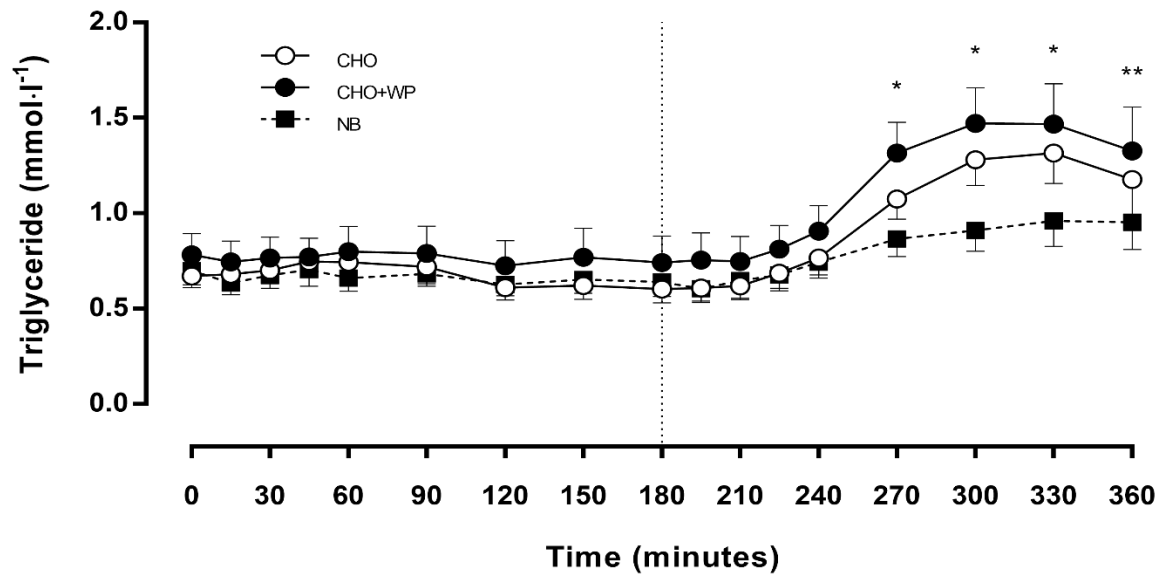


Figure 4.5 Mean \pm SEM temporal changes in plasma triglyceride concentrations throughout the full experimental protocol (0-360 minutes). Significant differences ($p < 0.05$) between trials at individual time points are defined as follows; *NB vs CHO+WP and CHO, **NB vs CHO+WP. CHO+WP: high-carbohydrate breakfast with added whey protein trial; CHO: high-carbohydrate breakfast trial; NB: no breakfast trial.

4.1.3.4 Continuous glucose monitoring

Interstitial glucose concentrations over 24 hours are displayed in Figure 4.6. Glycaemia and glycaemic variability were similar between CHO+WP and CHO over the 24 hour recording period (Table 4.4; $p > 0.05$). There were no differences in glycaemia between breakfast trials throughout, and glycaemia was similar between all trials over 24 hours and during the post-

laboratory period (6-24 hours AUC, Table 4.4; $p > 0.05$). During the laboratory protocol (0-6 hours), interstitial glucose AUC was significantly lower in NB than CHO+WP ($p = 0.043$), also appearing lower than CHO, although this comparison was not significant ($p = 0.182$). Glycaemic variability during the same laboratory period was greater in the CHO trial than NB ($p = 0.003$), however this measure did not differ between CHO+WP and CHO or NB ($p \geq 0.273$).

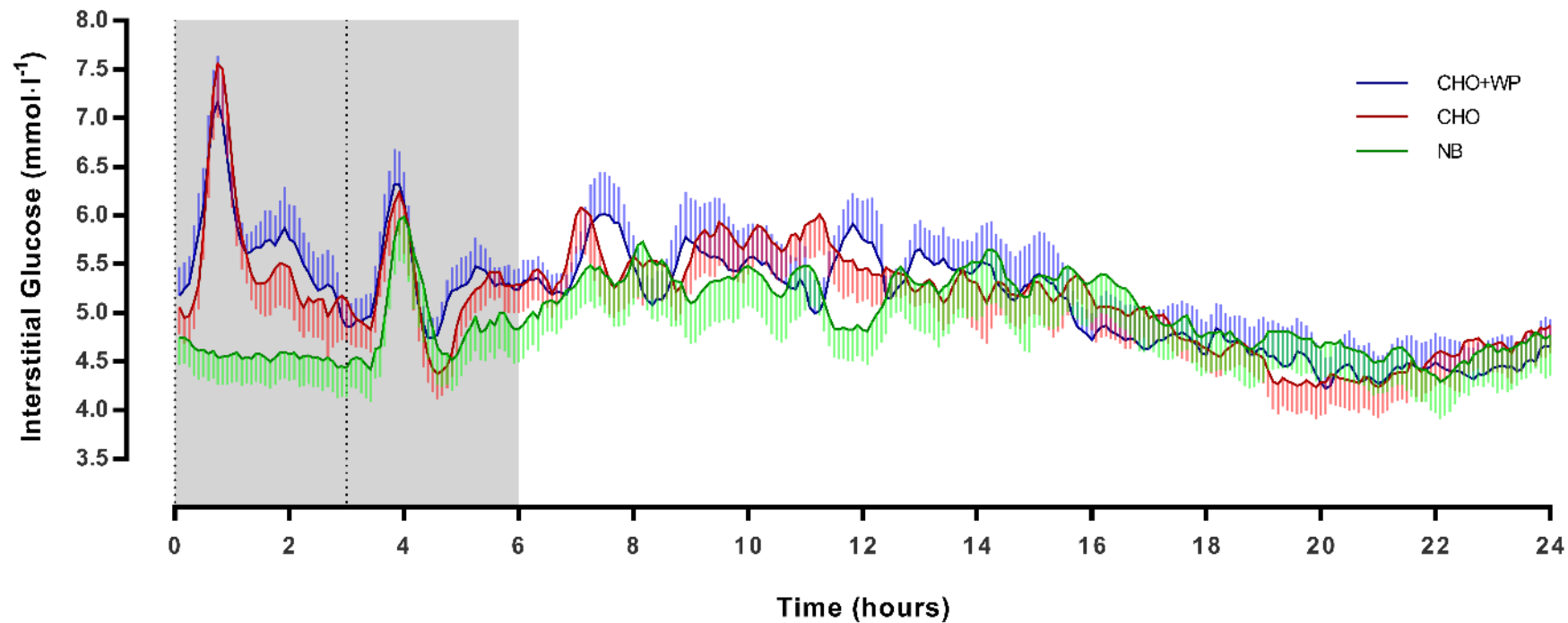


Figure 4.6 Interstitial glucose concentrations for 24 hours following test breakfast consumption. Data are presented as mean \pm SEM. Shaded area indicates time during laboratory protocol. Dotted lines indicate test meals. CHO+WP: high-carbohydrate breakfast with added whey trial; CHO: high-carbohydrate breakfast trial; NB: no breakfast trial.

Table 4.4 Area under the curve and coefficient of variation for interstitial glucose concentrations.

		Total (0-24 hours)	Post- Laboratory (6-24 hours)	Laboratory (0-6 hours)	Late afternoon (6-12 hours)	Night Time (12-18 hours)	Early Morning (18-24 hours)
Area under the curve (mmol*min·l⁻¹)	CHO+WP	7393 ± 340	5406 ± 279	1987 ± 94 ^a	1974 ± 103	1845 ± 111	1587 ± 107
	CHO	7420 ± 333	5480 ± 249	1940 ± 109 ^{a,b}	2027 ± 104	1856 ± 91	1598 ± 80
	NB	7141 ± 512	5429 ± 405	1712 ± 115 ^b	1897 ± 134	1888 ± 152	1644 ± 129
Coefficient of variation (%)	CHO+WP	16.7 ± 1.9	16.6 ± 1.9	11.9 ± 1.2 ^{a,b}	13.5 ± 1.2	11.8 ± 2.3	6.5 ± 0.8
	CHO	16.6 ± 1.4	15.8 ± 1.6	14.6 ± 1.3 ^a	11.9 ± 0.6	10.3 ± 1.3	12.1 ± 2.3
	NB	13.3 ± 0.8	12.9 ± 1.1	9.9 ± 1.3 ^b	11.7 ± 0.8	10.8 ± 1.5	7.6 ± 0.6

Data presented as mean ± SEM. Different superscript letters within a column indicates significant difference between conditions ($p < 0.05$) for each parameter presented. CHO+WP: high-carbohydrate breakfast with added whey trial; CHO: high-carbohydrate breakfast trial; NB: no breakfast trial.

4.1.4 Discussion

The aim of this study was to investigate the effect of supplementing a carbohydrate breakfast with whey protein on metabolic and appetite responses acutely and following a subsequent meal. The study was designed to reflect practical application, thus the amount of whey protein, the timing of supplementation and composition of meals were realistic in the context of habitual eating habits. Addition of 20 g whey protein to breakfast increased the acute insulin response, without significantly influencing glycaemia, lipaemia or appetite sensations compared to an identical meal without additional whey in healthy young males. Furthermore, for the first time it is shown that addition of whey protein to breakfast does not alter glycaemic, insulinaemic and appetite responses to a subsequent lunch meal, or glycaemia beyond this time, compared to breakfast without whey or fasting.

The observation of increased circulating insulin with whey protein supplementation reflects the effect observed in several studies (Gunnerud *et al.*, 2013; Nilsson *et al.*, 2007; Pal & Ellis, 2010). Potential mechanisms may centre on the amino acid profile of whey itself, and its consequent incretin hormone stimulatory properties (Jakubowicz & Froy, 2013). The synergistic effect of protein and carbohydrate co-ingestion is also well established (Nuttall *et al.*, 1985). Although a ~25% increase in post-breakfast (0-180 min) insulin AUC with the addition of whey to a carbohydrate breakfast was observed, this did not augment a significant effect on postprandial glycaemia. This is contrary to the findings of previous studies, where various doses of whey protein have elicited positive effects on glycaemia in both healthy (Akhavan *et al.*, 2014; Gunnerud *et al.*, 2013) and diabetic (Frid *et al.*, 2005; Ma *et al.*, 2009) populations. This may partly be explained by insulin resistance being greater early in the

morning following an overnight fast (Plat *et al.*, 1996), indicating that the insulinotropic effect was not strong enough to offset this. In addition, in contrast to the pure glucose loads used in previous whey supplementation studies (Gunnerud *et al.*, 2013; Nilsson *et al.*, 2007), a combination of carbohydrate sources was used in the present study as the test meal was designed to reflect habitually consumed carbohydrate-rich breakfast foods, resulting in a lower GI. The challenge to glucose tolerance may therefore not have been sufficiently large for a significantly augmented response to be observed. Whey protein ingestion has also been shown to induce acute insulin resistance through impaired glucose disposal at whole body level and across the leg (Smith *et al.*, 2015), albeit in obese post-menopausal female participants, however assessments of post-meal Matsuda-ISI were similar between breakfast trials in the current study. Whilst the mechanisms responsible for such an observation remain unclear, it is likely that any effects are counterbalanced by the promotion of insulin secretion by protein ingestion.

It is possible that the dose of whey protein was insufficient to elicit a significant reduction in glycaemia in healthy males, however the efficacious dose to affect insulin and post-meal glycaemic response has previously been reported to be as low as 10 g in healthy individuals (Akhavan *et al.*, 2010). Although post-breakfast glycaemia (in terms of AUC) was not significantly affected by whey protein addition to breakfast, the trend for reduced peak glucose, although not statistically significant in this participant group, may be indicative of a beneficial effect. The amplitude of postprandial glycaemic excursions, rather than simply mean glucose exposure, is recognised as an important factor in the advancement of declining metabolic health (Service, 2013), potentially via activation of oxidative stress (Monnier *et al.*, 2012). Following lunch, both glycaemia and insulinaemia were unaffected by prior whey

consumption, indicating the priming effect of elevated pre-lunch insulin on pancreatic beta cells may not have been substantial enough to alter post lunch metabolism (Lopez *et al.*, 2011). Glycaemia remained similar between all trials for 18 hours after leaving the laboratory. The lack of a second meal effect, or of any prolonged effect on glycaemia suggests that any influence of whey protein on postprandial metabolism is transient in nature, however further research may be required to establish whether this is the case in other populations.

In the present study, whey protein supplementation did not affect subjective appetite. Several studies have previously reported no effect of whey protein consumption on satiety and subsequent energy intake (Bowen *et al.*, 2007; Potier *et al.*, 2010). This contrasts with other published observations, where consumption of whey has enhanced satiety through a variety of proposed mechanisms, including increases in peak amino acid, GLP-1 and cholecystokinin (CCK) concentrations in healthy females (Hall *et al.*, 2003; Zafar *et al.*, 2013). As energy intake was not measured in the current study, it is difficult to infer any direct effect that appetite sensations may have had on subsequent intake (Blundell *et al.*, 2010).

Cross-sectional studies have associated regular breakfast consumption with improved health outcomes such as lower BMI and improved glycaemic control (Deshmukh-Taskar *et al.*, 2012; Kapantais *et al.*, 2011), however few randomised trials exist to support the causality of such associations. In the current study, breakfast omission did not affect postprandial glucose or insulin concentrations following lunch. With regards to satiety, breakfast omission did not significantly affect subjective appetite indices in the post lunch period and similar circulating concentrations of insulin, which is considered a satiety hormone (Tremblay &

Bellisle, 2015), reflected this. Further research is required to establish whether participants would overcompensate in terms of energy intake at a following meal regardless of subjective appetite ratings.

Measures of postprandial lipaemia were similar between both breakfast trials throughout, however the omission of breakfast appeared to impact on post meal lipid metabolism. NEFA was significantly higher following breakfast omission, likely mediated by the antilipolytic effects on HSL of insulin released in response to breakfast (Bano, 2013), and returned to similar levels to both breakfast trials 45 minutes after lunch. Elevated NEFA levels are associated with metabolic dysregulation and cardiovascular disease risk (Carlsson *et al.*, 2000). Plasma triglyceride concentrations were significantly lower 90-150 minutes post-lunch following breakfast omission. The very low fat content of the breakfast (2 g) would suggest that the handling of dietary fat from lunch (33 g) was affected by breakfast omission, or that fat stored in the enterocyte from the previous meal (evening before) was mobilised following subsequent glucose consumption (Lambert & Parks, 2012; Robertson *et al.*, 2003). This may have implications when considering the current swathe of public opinion suggesting that breakfast is the most important meal of the day. Our findings suggest that skipping breakfast on a single occasion may not be detrimental to the response to foods consumed later that day, and may provide beneficial effects on plasma triglyceride profile. Whether subsequent energy intake would be influenced remains to be established, however recent data indicates that the frequency, timing and composition of meals consumed later in the day are not different during 6 weeks of breakfast omission compared to regular breakfast consumption (Betts *et al.*, 2014).

The primary findings of the current study are contrary to the body of evidence which suggests whey protein can provide acute advantages in postprandial handling of meals. Acutely, the promotion of greater insulin secretion following whey protein consumption may lead to a glucose lowering effect (Gunnerud *et al.*, 2013; Nilsson *et al.*, 2007; Pal & Ellis, 2010). Chronically however, prolonged hyperinsulinaemia in normoglycaemic conditions may decrease insulin sensitivity (Del Prato *et al.*, 1994). The acute increase in circulating insulin observed in the present study may therefore be of benefit to individuals with type 2 diabetes, however the chronic effects in healthy, insulin sensitive individuals is less clear.

The breakfasts provided in the present study were not matched for energy content, and this may limit the validity of direct comparisons between each meal. This was a consequence however, of the effort to ensure that this experimental manipulation had practical application by mimicking the effect of ‘adding’ a supplement to a meal, rather than replacing or substituting different components of a meal. Such a strategy may be easier for a layperson to implement.

In summary, an ecologically valid protocol was implemented to assess the effect of supplementing a carbohydrate-rich breakfast with whey protein on metabolic and appetite responses, acutely and following a subsequent meal. Whey protein addition to breakfast increased the insulinaemic response to that meal, without significantly influencing metabolic or appetite responses to a subsequent mixed-macronutrient lunch. Breakfast omission induced glycaemic, insulinaemic and combined appetite responses to a subsequent meal that did not differ from breakfast consumption, while maintaining lower circulating triglyceride concentrations.

CHAPTER 4b

Acute and second-meal metabolic and appetite responses following co-ingestion of whey protein with a fat-rich breakfast in normal-weight males

4.2.1 Introduction

As discussed in Chapter 4a, the importance of the postprandial milieu in the assessment of CVD risk is increasingly recognised and, in addition to the clinically important risks associated with postprandial hyperglycaemia (Fonseca, 2003), postprandial hyperlipidaemia is considered an important independent determinant of CVD risk (Kolovou *et al.*, 2011; Silva *et al.*, 2005). The proatherogenic products of triglyceride-rich lipoprotein lipolysis are thought to mediate this risk (Ansar *et al.*, 2011), while elevated postprandial NEFA may contribute to the insulin resistant state (Frape *et al.*, 2000). Given that people spend the vast majority of non-sleeping hours in the postprandial state, it is important to investigate strategies which may reduce repeated exposure to exaggerated postprandial lipaemia.

It is well established that addition of carbohydrate to a fatty meal causes both a delay and a reduction in postprandial lipaemia, via alterations in absorption kinetics and modification of the endocrine response (Cohen & Berger, 1990; Westphal *et al.*, 2002). The influence of dietary protein in a fat-rich meal, in the context of postprandial lipaemia, is less well established (Westphal *et al.*, 2004). Epidemiological studies have indicated an inverse association between dairy protein intake and risk of developing CVD and metabolic disorders (Tremblay & Gilbert, 2009), however acute studies investigating this relationship are lacking.

The insulintrophic effect of whey protein supplementation has been established in previous studies (Gunnerud *et al.*, 2012a; Nilsson *et al.*, 2007) and was indicated in Chapter 4a. This property encourages speculation that addition of whey to high fat meals may have a beneficial effect on postprandial lipid exposure, initially through inhibition of NEFA mobilisation (Saponaro *et al.*, 2015) and subsequently via stimulated LPL-induced hydrolysis of triglyceride for metabolism or storage (Czech *et al.*, 2013). Currently, evidence from acute trials where high fat or mixed meals have been supplemented with whey protein is limited, and has shown inconsistency in findings. Consumption of 45 g whey protein isolate alongside a mixed macronutrient meal reduced the postprandial triglyceride AUC by 21% and 27% compared with glucose or casein supplementation respectively, in obese post-menopausal females (Pal *et al.*, 2010b). Whey has also been reported to decrease postprandial lipaemia compared with other protein sources when added to a fat-rich meal in participants with T2DM (Mortensen *et al.*, 2009). In overweight but otherwise healthy males, addition of whey to the same high fat breakfast increased triglyceride AUC compared with casein supplementation in one study (Mariotti *et al.*, 2015), but had a similar response to casein and favourable affects compared to non-dairy proteins in another study by the same group

(Holmer-Jensen *et al.*, 2013). To the author's knowledge, the effect of whey protein supplementation on postprandial lipaemia in normal-weight participants has not been investigated.

These studies have generally used test meals with an unrealistically high fat content (~80 g) to exceed the normal lipoprotein capacity (Holmer-Jensen *et al.*, 2013), along with relatively high supplemental doses of whey protein (45 g). However, to apply any findings to future dietary guidance, there is a need for studies investigating the efficacy of realistic doses of whey protein on levels of fat found within typical meals. Current studies have manipulated the macronutrient content of fat-rich test meals to compare the action of whey with other protein sources or energy-matched carbohydrate ingestion. It is unclear if the action of adding a dose of whey protein alongside a high fat meal to compare against the effect of just the meal itself, a potentially more pragmatic approach, would induce beneficial postprandial responses. Furthermore, studies have typically investigated responses to an isolated meal by evaluating postprandial markers of lipaemia over extended evaluation periods of six (Mariotti *et al.*, 2015; Pal *et al.*, 2010b) or eight (Holmer-Jensen *et al.*, 2013; Mortensen *et al.*, 2009) hours. However, given that a mean interval between eating episodes of ~3 hours has been documented in cross-sectional analysis of American adults (Kant & Graubard, 2015), it is unlikely that such an extended period is commonplace in a free-living setting. Thus the use of sequential meal testing is of interest, particularly regarding triglyceride metabolism, where it remains to be established if prior whey protein supplementation with a fat-rich meal influences metabolic responses to subsequent meals. In addition, it is unclear whether the potential of whey protein to increase satiety (documented previously (Luhovyy *et al.*, 2007; Veldhorst *et al.*, 2009) but not in Chapter 4a) is observed when co-ingested with high-fat

rather than high-carbohydrate test meals. Therefore, this study aimed to determine the acute and second meal postprandial metabolic and appetite responses in normal-weight individuals following a fat-rich breakfast, with and without co-ingestion of whey protein.

4.2.2 Methods

4.2.2.1 Experimental design

The experimental protocol described in this sub-chapter is a replication of that detailed in Chapter 4a. As described in section 3.2, analysis of two additional experimental trials is presented here, together with data from the control trial. The same ten normal-weight male participants (age: 24 ± 1 years; mass: 79.7 ± 1.2 kg; stature: 1.81 ± 0.02 m; BMI: 24.5 ± 0.7 kg·m⁻², HOMA-IR: 2.1 ± 1.1 arbitrary units) consumed a fat-rich breakfast with (FAT+WP) or without (FAT) supplemental whey protein, or omitted breakfast (NB). Other than the difference in test breakfast content, all other protocols are as described in Chapter 4a. A schematic of the laboratory protocol is presented in Figure 4.7.

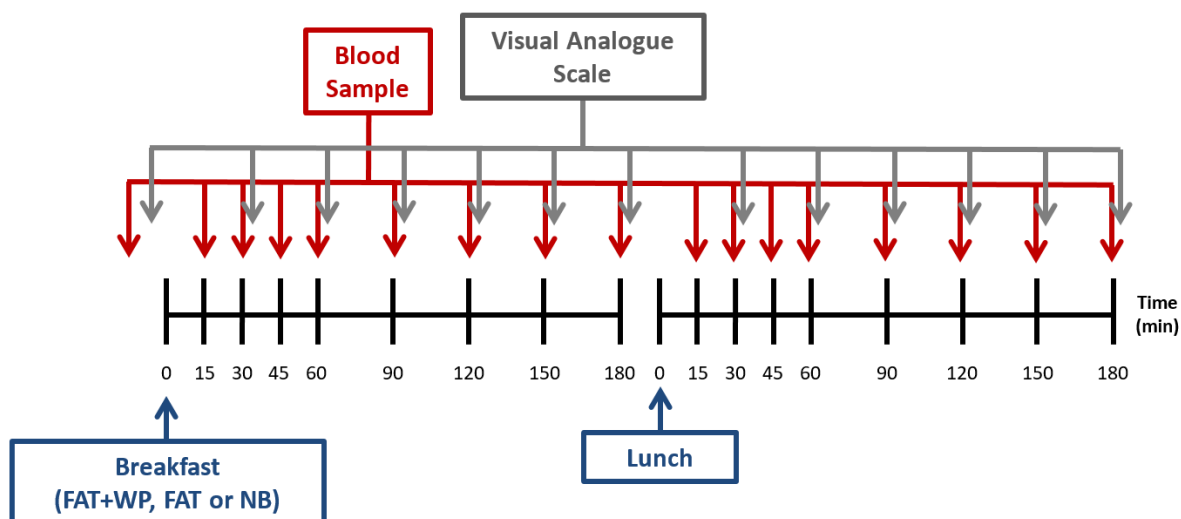


Figure 4.7 Schematic layout of laboratory experimental protocol. FAT, high fat breakfast trial; FAT+WP, high-fat breakfast with added whey protein trial; NB, no breakfast trial.

4.2.2.2 Test meals

The energy content and macronutrient composition of the test meals provided during each trial are displayed in Table 4.5. The breakfast meal given under FAT and FAT+WP consisted of egg yolk (60 g) mixed with single cream (75g) and butter (10g) until homogenous, and heated in a microwave (1,000 W) for 150 seconds, resulting in a semi-solid portion of cooked eggs. A beverage containing 20 g available protein (FAT+WP) or isovolumetric flavoured water (FAT) was served alongside the breakfast meal as described in section 4.1.2.3.

Table 4.5 Energy and macronutrient composition of breakfast and lunch test meals.

		Energy kJ (kcal)	Carbohydrate g (E%)	Fat g (E%)	Protein g (E%)
Breakfast	FAT	1831 (438)	4 (4)	41 (85)	12 (11)
	FAT+WP	2205 (527)	6 (5)	41 (71)	32 (24)
	NB	0 (0)	0 (0)	0 (0)	0 (0)
Lunch		3446 (824)	104 (50)	33 (36)	29 (14)

E%: percentage of total meal energy; FAT: high-fat breakfast trial; FAT+WP: high-fat breakfast with added whey protein trial; NB: no breakfast trial.

4.2.2.3 Statistical analysis

Data were analysed as described in Chapter 4a (section 4.1.2.7).

4.2.3 Results

4.2.3.1 Plasma triglyceride

Plasma triglyceride did not differ between conditions at baseline ($p > 0.05$). There were significant main effects of time ($p < 0.001$), condition ($p = 0.001$), and a condition x time interaction ($p < 0.001$) on triglyceride responses. Post hoc analysis revealed that concentrations were higher for FAT+WP ($p = 0.008$) and FAT ($p = 0.017$) compared to NB (Figure 4.8), also reflected in significantly higher peaks for the breakfast trials (FAT+WP: 2.59 ± 0.35 , FAT: 2.24 ± 0.29 vs NB: 1.05 ± 0.13 mmol·l⁻¹; both $p = 0.001$), with no difference between FAT+WP and FAT throughout ($p > 0.05$). Concentrations rose over time

during FAT+WP and FAT, peaking at 225 minutes post-breakfast under both conditions, but did not significantly differ from baseline in NB ($p > 0.05$).

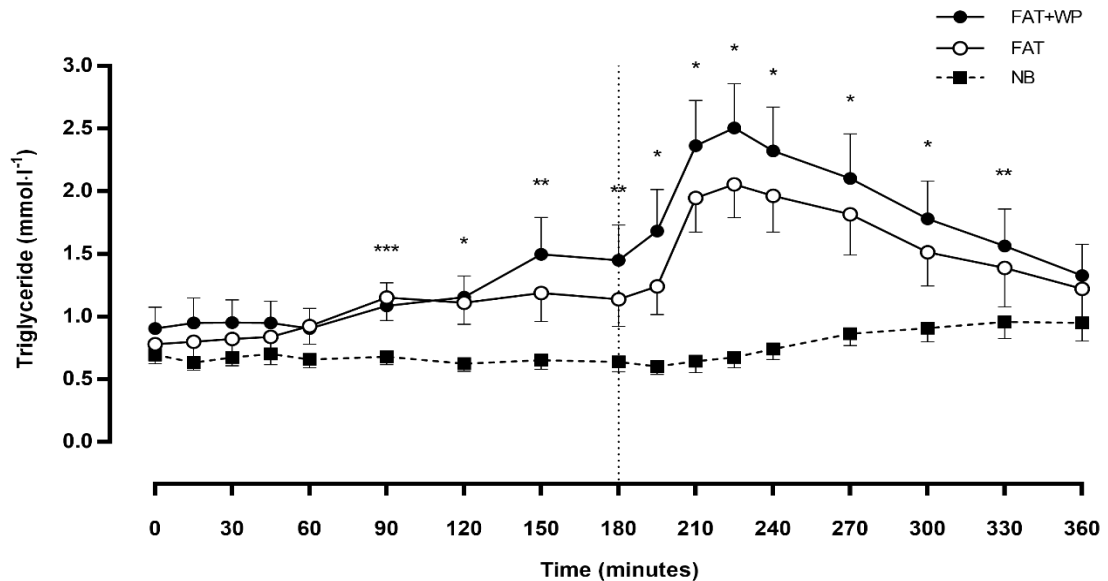


Figure 4.8 Mean \pm SEM ($n = 10$) temporal changes in plasma triglyceride concentrations throughout the full experimental protocol (0-360 minutes). Significant differences ($p < 0.05$) between trials are denoted as follows; *NB vs FAT+WP and FAT, **NB vs FAT+WP, ***NB vs FAT; Dotted line indicates time of lunch meal; FAT+WP: high-fat breakfast with added whey protein trial; FAT: high-fat breakfast trial; NB: no breakfast trial.

Post-breakfast triglyceride AUC in the FAT+WP condition was significantly greater than NB ($p = 0.039$) but not FAT ($p > 0.05$), while FAT AUC displayed a trend to be higher than NB ($p = 0.071$), but was not significant after correcting for multiple comparisons (Table 4.6). Following lunch, and over the entire evaluation period, AUC was greater for FAT+WP and FAT conditions compared with NB (all $p \leq 0.018$).

Table 4.6 Area under the curve for blood and plasma variables during post-breakfast (0-180 minutes) and post-lunch (180-360 minutes) postprandial periods.

		Area under the curve		
		FAT+WP	FAT	NB
Triglyceride (mmol·min ⁻¹ ·l ⁻¹)	Post-breakfast	204.2 ± 36.1 ^a	184.4 ± 30.5 ^{a,b}	118.8 ± 12.2 ^b
	Post-lunch	345.0 ± 56.2 ^a	291.6 ± 48.4 ^a	146.8 ± 17.4 ^b
	Total	549.2 ± 91.6 ^a	476.0 ± 78.3 ^a	265.6 ± 29.1 ^b
NEFA (mmol·min ⁻¹ ·l ⁻¹)	Post-breakfast	50.6 ± 6.1	68.5 ± 9.2	79.7 ± 14.1
	Post-lunch	88.8 ± 6.5 ^a	92.7 ± 10.7 ^a	37.5 ± 3.9 ^b
Glycerol (μmol·min ⁻¹ ·l ⁻¹)	Post-breakfast	9445 ± 1336	8180 ± 918	10244 ± 1702
	Post-lunch	11697 ± 1116 ^a	12376 ± 1572 ^{a,b}	7243 ± 1193 ^b
Glucose (mmol·min ⁻¹ ·l ⁻¹)	Post-breakfast	702.8 ± 17.9	726.7 ± 17.6	760.6 ± 25.7
	Post-lunch	817.2 ± 21.9 ^a	815.8 ± 29.8 ^a	744.2 ± 28.3 ^b
Insulin (pmol·min ⁻¹ ·l ⁻¹)	Post-breakfast	16475 ± 2085 ^a	13186 ± 1792 ^a	8306 ± 1446 ^b
	Post-lunch	29946 ± 2556 ^a	25560 ± 2887 ^b	19935 ± 3349 ^c

Data presented as mean ± SEM. Different superscript letters within a row indicates significant difference between conditions ($p < 0.05$). FAT+WP: high-fat breakfast with added whey protein trial; FAT: high-fat breakfast trial; NB: no breakfast trial; NEFA: non-esterified fatty acids.

4.2.3.2 Post-breakfast responses

Plasma NEFA displayed a main effect of time ($p < 0.01$) and a condition x time ($p < 0.01$) interaction indicating a differential time response between trials. Concentrations did not differ significantly from baseline levels in FAT and NB trials ($p > 0.05$), but were suppressed at 45-90 minutes post consumption of whey protein with breakfast ($p \leq 0.029$), before returning to pre-breakfast levels (Figure 4.9a). There were no between-condition differences in plasma NEFA at individual time points or in AUC across the post-breakfast period (Table 4.6; $p > 0.05$). Plasma glycerol was significantly affected by time ($p = 0.001$), such that

concentrations were suppressed in the postprandial period in all conditions, before returning to baseline levels (Figure 4.9b). No between-condition differences were observed, and AUC was similar across trials during this period (Table 4.6; $p > 0.05$).

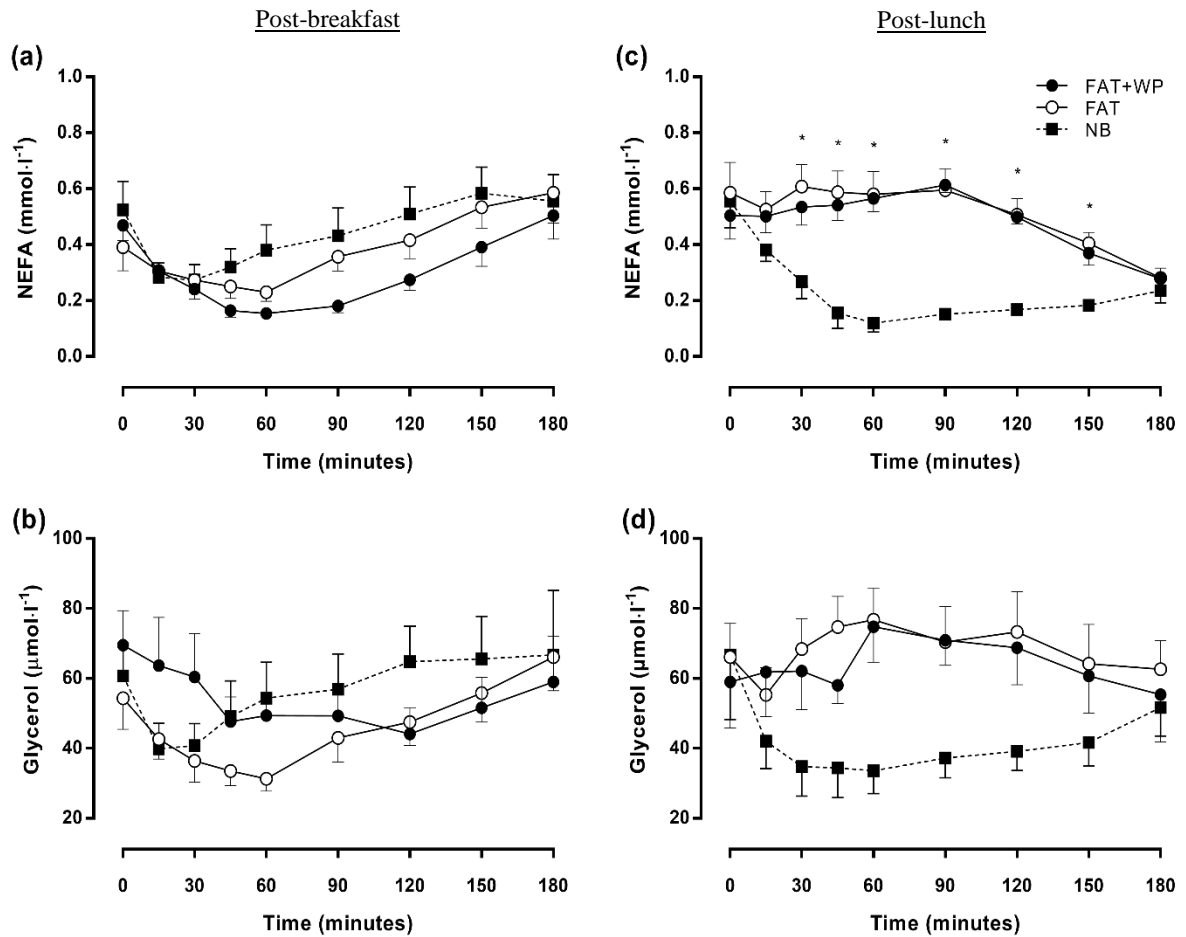


Figure 4.9 Mean \pm SEM temporal changes in NEFA (a) and glycerol (b) during the post-breakfast period (0-180 minutes), and NEFA (c) and glycerol (d) during the post-lunch period (180-360 minutes). Significant differences ($p < 0.05$) between trials at individual time points are defined as follows; *NB vs FAT+WP and FAT. FAT+WP: high-fat breakfast with added whey protein trial; FAT: high-fat breakfast trial; NB: no breakfast trial; NEFA: non-esterified fatty acids.

Post-breakfast glucose concentrations displayed a significant main effect of time ($p < 0.001$) and a condition \times time interaction effect ($p = 0.001$). Concentration did not change significantly from baseline level in the NB trial ($p > 0.05$), however levels were reduced compared with baseline at 45-120 minutes following breakfast consumption in FAT+WP (all $p \leq 0.011$) and at 60 minutes post in FAT ($p = 0.004$), before returning to pre-breakfast levels (Figure 4.10a). Glucose was significantly lower in the whey protein trial than FAT and NB at 45 minutes following breakfast ($p \leq 0.031$), and remained lower than NB at 60 minutes post ($p = 0.019$). Differences between trials in blood glucose AUC were not observed, although the main effect approached significance (Table 4.6; $p = 0.83$).

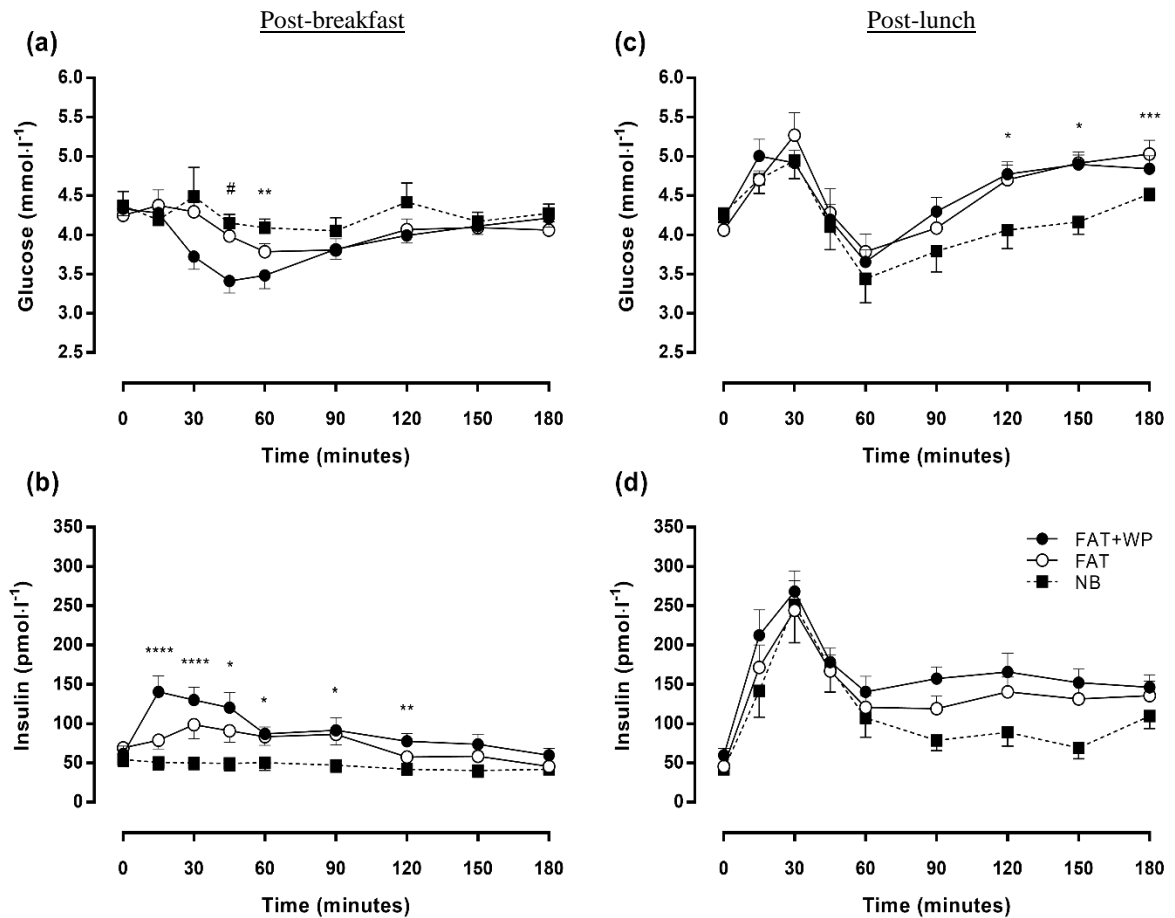


Figure 4.10 Mean \pm SEM temporal changes in glucose (a) and insulin (b) during the post-breakfast period (0-180 minutes), and glucose (c) and insulin (d) during the post-lunch period (180-360 minutes). Significant differences ($p < 0.05$) between trials at individual time points are defined as follows; *NB vs FAT+WP and FAT, **NB vs FAT+WP, ***NB vs FAT, ****All trials differ, #FAT+WP vs FAT and NB. FAT+WP: high-fat breakfast with added whey protein trial; FAT: high-fat breakfast trial; NB: no breakfast trial.

Two-way ANOVA revealed significant main effects of condition ($p < 0.001$), time ($p < 0.001$) and an interaction effect ($p < 0.001$) on plasma insulin levels (Figure 4.10b). Peak concentration was significantly greater following breakfast with (FAT+WP) compared to without (FAT) whey protein ($p = 0.004$), and greater in both breakfast conditions compared to NB (FAT+WP: 157.9 ± 18.8 ; FAT: 105.9 ± 17.1 vs NB: 65.8 ± 9.3 pmol.l⁻¹; both

$p \leq 0.009$). Insulin was unchanged throughout this period in NB ($p > 0.05$), but rose after breakfast in FAT+WP and FAT, remaining elevated above NB at 15-120 minutes in FAT+WP (all $p \leq 0.006$) and 15-90 minutes in FAT (all $p \leq 0.013$). A significant difference in AUC across the postprandial period was observed, such that insulinaemia was greater in FAT+WP ($p = 0.003$) and FAT ($p = 0.001$) compared to NB (Table 4.6). During the first 60 minutes post-breakfast, insulin AUC was also greater in FAT+WP ($6984 \pm 829 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{l}^{-1}$) than FAT ($5170 \pm 793 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{l}^{-1}$; $p = 0.008$). Matsuda-ISI was similar between breakfast conditions in the post-breakfast period (FAT+WP: 13 ± 2 , FAT: 14 ± 2 arbitrary units; $p = 0.356$).

Combined appetite score showed significant main effects for time ($p < 0.001$) and condition ($p < 0.001$), and a condition x time interaction effect ($p < 0.001$) was present. In FAT+WP and FAT conditions, combined appetite decreased to similar levels at 30 minutes following breakfast consumption, indicating greater satiety, before increasing similarly at 30-180 minutes (Figure 4.11a). In NB, combined appetite was similar to other trials at baseline and increased steadily over the postprandial period, with levels significantly greater than FAT+WP ($p = 0.001$) and FAT ($p < 0.001$). Appetite AUC was significantly different between NB and both FAT+WP and FAT (all $p < 0.05$) for all aspects measured (hunger, fullness, PFC, satisfaction), with breakfast consumption impacting positively on appetite perceptions (Table 4.7). There were no differences between FAT+WP and FAT throughout ($p > 0.05$).

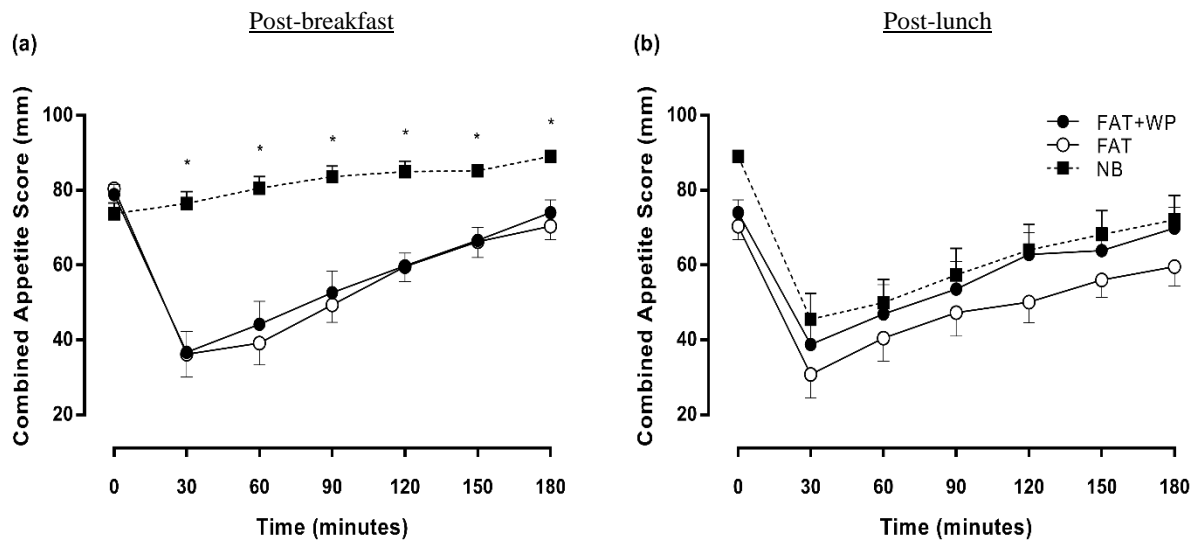


Figure 4.11 Mean \pm SEM temporal changes in combined appetite score during the post-breakfast (a) and post-lunch (b) periods. Significant differences ($p < 0.05$) between trials at individual time points are defined as follows; *NB vs FAT+WP and FAT. FAT+WP: high-fat breakfast with added whey protein trial; FAT: high-fat breakfast trial; NB: no breakfast trial.

Table 4.7 Area under the curve for subjective appetite responses during the post-breakfast (0-180 minutes) and post-lunch (180-360 minutes) postprandial periods.

		Area under the curve		
		FAT+WP	FAT	NB
Combined appetite (mm·min ⁻¹)	Post-breakfast	10093 \pm 697 ^a	9781 \pm 702 ^a	14768 \pm 443 ^b
	Post-lunch	10142 \pm 1142 ^{a,b}	8691 \pm 912 ^a	10971 \pm 1024 ^b
Hunger (mm·min ⁻¹)	Post-breakfast	9241 \pm 644 ^a	9163 \pm 718 ^a	13406 \pm 570 ^b
	Post-lunch	9494 \pm 1031 ^{a,b}	8046 \pm 976 ^a	10307 \pm 969 ^b
Fullness (mm·min ⁻¹)	Post-breakfast	8209 \pm 691 ^a	8447 \pm 726 ^a	2853 \pm 431 ^b
	Post-lunch	8186 \pm 1224 ^{a,b}	9447 \pm 898 ^a	7061 \pm 1033 ^b
PFC (mm·min ⁻¹)	Post-breakfast	11125 \pm 890 ^a	10771 \pm 923 ^a	15296 \pm 435 ^b
	Post-lunch	11241 \pm 1258 ^{a,b}	9741 \pm 1080 ^a	11780 \pm 1143 ^b
Satisfaction (mm·min ⁻¹)	Post-breakfast	7785 \pm 632 ^a	8365 \pm 608 ^a	2775 \pm 454 ^b
	Post-lunch	7982 \pm 1129 ^{a,b}	9578 \pm 785 ^a	7139 \pm 986 ^b

Data presented as mean \pm SEM. Different superscript letters within a row indicates significant difference between conditions ($p < 0.05$). FAT+WP: high-fat breakfast with added whey protein trial; FAT: high-fat breakfast trial; NB: no breakfast trial; PFC: prospective food consumption.

4.2.3.3 Post-lunch responses

Post-lunch NEFA levels were influenced significantly by time ($p < 0.01$) and trial ($p < 0.01$), with two-way ANOVA also indicating an interaction effect of these two factors ($p < 0.01$). A suppression of plasma NEFA was observed following lunch in NB, such that values were significantly lower than baseline at 30-60 minutes post-lunch ($p \leq 0.014$), however levels remained unchanged in FAT+WP and FAT (Figure 4.9c; $p > 0.05$). Post hoc analysis showed that plasma NEFA concentrations were greater in both breakfast trials in comparison to NB at 30-150 minutes post-lunch ($p < 0.05$). Furthermore, post-lunch AUC was greater for FAT+WP ($p < 0.001$) and FAT ($p = 0.001$) in comparison to NB (Table 4.6).

There was a significant effect of trial on post-lunch plasma glycerol concentrations ($p = 0.044$). Post hoc analysis revealed that glycerol was reduced in NB in comparison to FAT+WP (Figure 4.9d; $p = 0.032$). Post-lunch AUC was also significantly lower in NB compared to FAT+WP ($p = 0.01$), while the difference between FAT and NB approached significance (Table 4.6; $p = 0.089$). There were no differences between FAT+WP and FAT throughout ($p > 0.05$).

Time ($p < 0.001$), condition ($p = 0.021$) and time x condition interaction ($p = 0.031$) effects were observed on blood glucose concentrations following lunch. Glucose peaked similarly at 30 minutes post-lunch ($p > 0.05$), however responses were divergent later in the postprandial period such that glucose was reduced in NB compared to FAT at 120-180 minutes ($p \leq 0.043$), and compared to FAT+WP at 120-150 minutes post-lunch (Figure 4.10c;

$p \leq 0.023$). Blood glucose AUC was similar between FAT+WP and FAT ($p > 0.05$), both significantly greater over the post-lunch period than NB (Table 4.6; $p \leq 0.019$).

Post-lunch analysis revealed main effects of both time ($p < 0.001$) and condition ($p < 0.001$) on plasma insulin concentrations. Levels peaked at 30 minutes post-lunch and remained elevated above baseline throughout the postprandial period in all trials (Figure 4.10d). Concentrations were higher following both breakfast trials compared to NB (both $p \leq 0.028$), and the increase in FAT+WP compared with FAT was close to significance ($p = 0.058$). Analysis of post-lunch AUC revealed that insulinaemia was greater in FAT+WP than FAT ($p = 0.035$), and higher in both compared with NB (Table 4.6; $p \leq 0.005$). Greater insulin sensitivity was observed in the non-whey breakfast trial (Matsuda-ISI: FAT+WP: 9 ± 1 , FAT: 11 ± 2 arbitrary units; $p = 0.037$). After prior breakfast omission, post-lunch insulin sensitivity (NB: 16 ± 3 arbitrary units) was greater than FAT+WP ($p = 0.043$) and tended to be greater than FAT ($p = 0.079$) although this comparison was not significant after correcting for multiple comparisons.

Subjective appetite responses were influenced by time ($p < 0.001$), such that appetite was suppressed under all conditions at 30 minutes post-lunch, rising steadily thereafter, with responses back at similar levels to baseline by 150 minutes (Figure 4.11b). A main effect of trial was observed ($p = 0.002$), with subjective appetite suppressed to a greater extent in FAT compared to NB ($p = 0.002$), but not FAT+WP ($p = 0.351$). Post hoc analysis of significant differences in AUC for component measures of appetite (hunger, fullness, PFC, satisfaction) and combined appetite revealed more positive perceptions of appetite in the FAT trial

compared with NB ($p < 0.05$), with no differences between FAT and FAT+WP (Table 4.7; $p > 0.05$).

4.2.3.4 Continuous glucose monitoring

Due to technical issues with a CGM device for one participant during the FAT trial, the following data are presented as $n = 9$. Interstitial glucose concentrations over 24 hours are displayed in Figure 4.12. Glucose AUC over 24 hours was similar between FAT+WP and FAT ($p > 0.05$), however glycaemia during this period was lower when breakfast was omitted in comparison to both breakfast trials (both $p \leq 0.043$; Table 4.8). When analysis was conducted on separate 6 hour epochs, no between-trial differences in glycaemia were observed ($p > 0.05$), however there were trends for glycaemia to be reduced during the 6 hour laboratory period in NB compared to FAT+WP ($p = 0.077$), as well as over the 18 hour post-laboratory period in NB compared to both FAT+WP ($p = 0.081$) and FAT ($p = 0.061$) trials. Glycaemic variability was similar between conditions throughout.

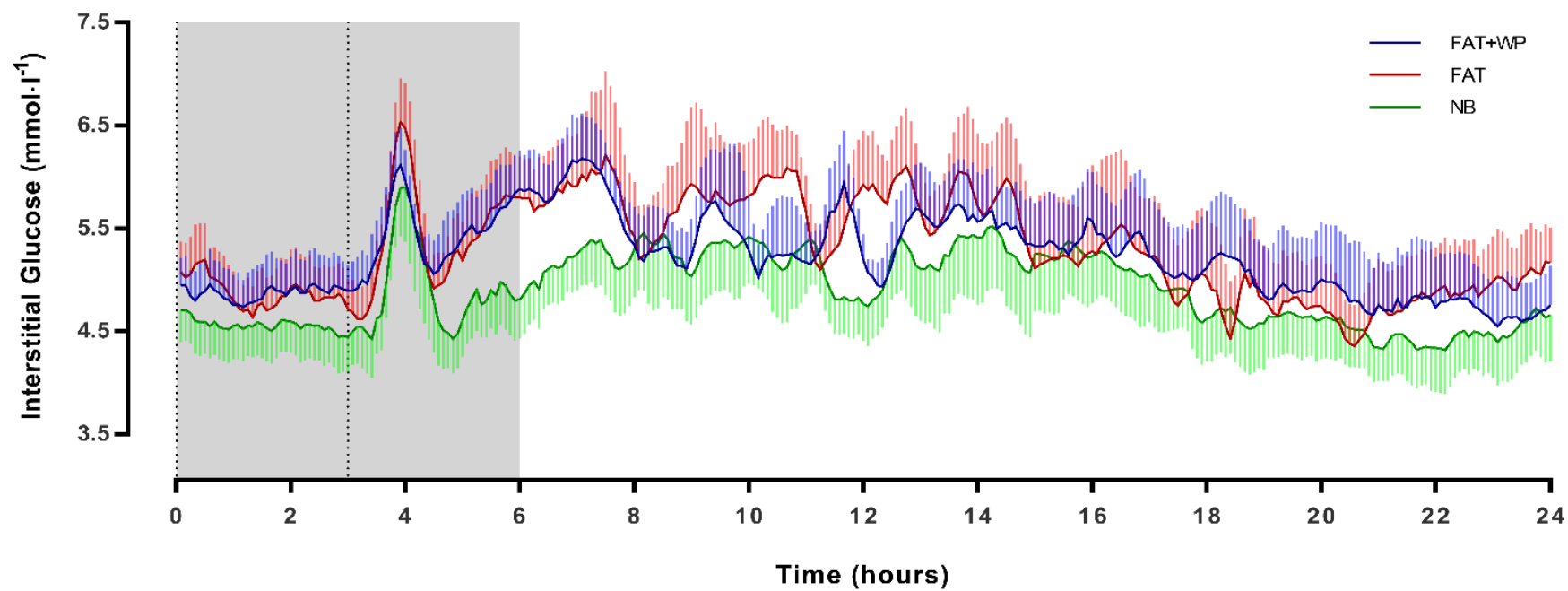


Figure 4.12 Interstitial glucose concentrations for 24 hours following test breakfast consumption. Data are presented as mean \pm SEM. Shaded area indicates time during laboratory protocol. Dotted lines indicate test meals. FAT+WP: high-fat breakfast with added whey protein trial; FAT: high-fat breakfast trial; NB: no breakfast trial.

Table 4.8 Area under the curve and coefficient of variation for interstitial glucose concentrations.

		Total (0-24 hours)	Post- Laboratory (6-24 hours)	Laboratory (0-6 hours)	Late afternoon (6-12 hours)	Night Time (12-18 hours)	Early Morning (18-24 hours)
Area under the curve (mmol*min·l⁻¹)	FAT+WP	7502 ± 536 ^a	5642 ± 429	1859 ± 114	1993 ± 138	1933 ± 137	1717 ± 171
	FAT	7601 ± 592 ^a	5742 ± 485	1859 ± 118	2069 ± 172	1968 ± 201	1705 ± 149
	NB	7040 ± 561 ^b	5335 ± 441	1705 ± 128	1872 ± 147	1854 ± 166	1609 ± 139
Coefficient of variation (%)	FAT+WP	14.6 ± 1.5	15.5 ± 1.8	9.5 ± 1.0	12.6 ± 0.9	14.5 ± 2.2	7.9 ± 1.3
	FAT	16.8 ± 1.6	17.1 ± 1.7	11.6 ± 1.1	12.6 ± 0.9	13.1 ± 2.1	10.7 ± 2.2
	NB	13.2 ± 0.8	13.0 ± 1.2	9.7 ± 1.4	11.4 ± 0.8	10.9 ± 1.6	7.2 ± 0.6

Data presented as mean ± SEM. Different superscript letters within a column indicates significant difference between conditions ($p < 0.05$) for each parameter presented. FAT+WP: high-fat breakfast with added whey protein trial; FAT: high-fat breakfast trial; NB: no breakfast trial.

4.2.4 Discussion

The main findings of the current study are that a 20 g dose of whey protein augmented the insulin response both acutely (within the first hour) and following a second meal, however postprandial lipaemia was not significantly affected by the intervention. In addition, glycaemia was similar between breakfast conditions over the course of the laboratory protocol and during the rest of the day, while perceptions of appetite were unaffected by whey protein supplementation.

Postprandial triglyceride responses peaked at 3-4 hours following a fat-rich breakfast, emphasising the relatively slow appearance of dietary fat into the plasma (Lambert & Parks, 2012), with a greater response in breakfast trials compared to breakfast omission, as would be expected. In contrast to the findings of Pal *et al.* (2010b), where supplemental whey protein induced a 21% reduction in postprandial triglyceride AUC following a high fat meal, there was no modulation of postprandial lipaemia following consumption of whey with breakfast in the present study. The magnitude of this reduction reported by Pal *et al.* (2010b) may be considered clinically significant since a 20-24% reduction in triglyceride reduces the progression of CVD (Miller, 2000), and similar decrements have been observed in further studies (Holmer-Jensen *et al.*, 2013; Mortensen *et al.*, 2009).

The discrepancy when comparing such findings to the present study may be explained by fundamental differences in study design. This study was the first to investigate the effect of whey protein on postprandial lipaemia in healthy, normal-weight individuals, whereas previous investigations have involved obese (Holmer-Jensen *et al.*, 2013; Pal *et al.*, 2010b)

or diabetic (Mortensen *et al.*, 2009) participants. The negative effects of a high fat meal that are associated with postprandial hypertriglyceridaemia, including oxidative stress, low-grade inflammation and endothelial dysfunction, are considerably more pronounced when participants present with risk factors for metabolic disease such as obesity (Mariotti *et al.*, 2015). Indeed, both the increment and duration of postprandial lipaemia are exaggerated in obese compared to lean individuals (van Wijk *et al.*, 2003), and it may therefore be the case that plasma triglyceride is not as sensitive to such an intervention when responses are already in the normal range. Moreover, a recent meta-analysis of longer term interventions of chronic whey protein supplementation reported that significant reductions in triglyceride were not observed in participants with a BMI < 30 kg·m⁻² (Zhang *et al.*, 2016). Interestingly, the same study reported that effects were not observed when less than 30 g whey protein was supplemented per day, indicating that protein amount may be significant when investigating the beneficial effects of whey protein on triglyceride concentrations. The studies mentioned above all supplemented meals with 45 g whey protein, however the current study was designed to assess the effects of a more realistic dose (20 g) of whey protein. This amount has been shown to be efficacious in reducing glycaemia in previous studies (Abou-Samra *et al.*, 2011; Akhavan *et al.*, 2010), however a greater stimulus may be required to positively influence lipid metabolism. Fat content of test meals (80 g) implemented by Holmer-Jensen *et al.* (2013) and Mortensen *et al.* (2009) was also approximately double that used in the present study, indicating that the fat-rich meal administered may not have challenged fat tolerance in healthy individuals to a sufficient extent for beneficial effects to be observed.

Previous studies have assessed the effectiveness of whey protein to reduce postprandial lipaemia compared to other proteins or energy-matched amount of glucose. The current study

was designed to assess the metabolic response when whey was added to a fat-rich meal compared to the response of the meal itself. Such an approach using casein protein has previously induced beneficial effects on postprandial lipid metabolism in healthy individuals, including marked suppression of NEFA and moderate reduction in postprandial triglyceride (Westphal *et al.*, 2004). The reduction in lipaemia was only observed when casein was supplemented to the high-fat meal in the presence of carbohydrate, an effect also observed by Brader *et al.* (2010) in diabetic participants. This is suggestive of a role of carbohydrate coingestion with dairy protein on postprandial lipaemic responses, indeed studies that have supplemented whey in this context have all included carbohydrate (25-45 g) in the high-fat test meals (Holmer-Jensen *et al.*, 2013; Mortensen *et al.*, 2009; Pal *et al.*, 2010b). The test meal in this study included a low carbohydrate content (4 g) to isolate the effects of whey protein on subsequent metabolism following a fat-rich meal. Carbohydrate and protein act synergistically to augment the postprandial insulin response (Nuttall *et al.*, 1985). Although insulin was increased following whey consumption, the stimulus may not have been great enough to influence lipaemia or other aspects of postprandial metabolism including glycaemia and appetite.

Proposed mechanisms by which whey protein may modulate the postprandial lipaemic response relate to enhanced insulinaemia. The inhibitory effect of insulin on intracellular HSL markedly suppresses the release of NEFA into the circulation (Saponaro *et al.*, 2015), limiting the supply of NEFA to the liver and subsequently slowing hepatic triglyceride synthesis and very low density lipoprotein (VLDL) secretion (Westphal *et al.*, 2004). The hydrolysis of triglyceride in chylomicrons and VLDL is augmented by the activity of LPL which may be stimulated by insulin (Czech *et al.*, 2013), resulting in accelerated clearance

of triglyceride-rich lipoproteins. Post-breakfast insulin AUC was significantly greater in the first hour following breakfast and, although not significant, ~25% greater over 3 hours when whey was added to the fat-rich breakfast. This is indicative of the insulinotropic effect of whey (Nilsson *et al.*, 2007). Despite this increase, the macronutrient composition of the breakfast ensured relatively low absolute postprandial insulin concentrations across both breakfast conditions. Post-breakfast peak insulin was $158 \text{ pmol}\cdot\text{l}^{-1}$ in the FAT+WP condition, considerably lower than when carbohydrate was co-ingested with whey protein in Chapter 4a ($447 \text{ pmol}\cdot\text{l}^{-1}$), while AUC was approximately 2-fold higher following the mixed macronutrient lunch in comparison with the fat-rich breakfast. Post-breakfast insulinaemia may therefore have been insufficient to augment significant changes in lipid metabolism.

Post-breakfast NEFA was transiently suppressed from baseline concentration following whey consumption, however levels were not significantly different to the other trials throughout, indicating that the increased insulin effect observed during this period did not markedly influence NEFA suppression. Following lunch, NEFA concentrations were similar in both breakfast trials, despite greater insulinaemia following whey consumption. A similar effect was observed in post-lunch glycaemia, which may be indicative of temporary peripheral insulin resistance, indeed Matsuda-ISI differed significantly between these conditions following lunch, thus it cannot be ruled out that the greater insulin levels observed were a result of amino acid induced insulin resistance (Smith *et al.*, 2015). Insulin resistance is associated with impaired clearance and increased production of chylomicrons (Pal *et al.*, 2010b) which may increase triglyceride exposure. Although a non-significant observation, total triglyceride AUC was ~15% greater in the FAT+WP condition in comparison with FAT, which may be explained by this phenomenon.

Greater post-lunch insulin sensitivity was observed following omission of breakfast, illustrated by reduced glycaemia with concomitantly lower insulin in comparison to breakfast trials. Insulin remained elevated above baseline for the entire 3 hour period in all trials however, and in line with the findings of Westphal *et al.* (2004), glucose levels were not responsive to this increase, since it is clear that concentrations were rising at the end of the experimental protocol which is confirmed in interstitial glucose data. Post-lunch NEFA appeared to be suppressed similarly in breakfast trials, however there was a marked delay in suppression when compared to breakfast omission. It may be concluded that the effect of elevated post-lunch NEFA after a fat-rich breakfast is a result of spillover of NEFA into the plasma from hydrolysis of chylomicrons, since only approximately half is taken up by adipocytes (Karpe *et al.*, 2011). However, the lack of suppression of glycerol, a marker of adipocyte lipolysis, would suggest a lack of insulin action on adipose lipolysis following the second meal, independent of protein supplementation.

The findings of the present study appear to present a mixed picture surrounding the effects of whey protein on postprandial metabolic responses following a high-fat meal. Previous studies have outlined potentially beneficial effects on postprandial lipaemia in obese individuals or those with T2DM, however similar observations were not found in healthy normal-weight young males in the present study, potentially due to the lack of a challenge to the already greater fat tolerance in such individuals. The omission of breakfast had a greater effect on post-lunch metabolic responses than the modulation of meal macronutrient intake. Post-lunch glucose, insulin and NEFA concentrations were reduced following prior breakfast omission, despite similar pre-lunch levels to breakfast trials. Triglyceride was also significantly lower during this period due to the post-breakfast peak occurring within this

time in breakfast trials, also accentuated by the second meal effect, whereby appearance of fat from a previous meal is accelerated by consumption of a subsequent meal (Lambert & Parks, 2012). Such findings appear to counter the negative connotations associated with breakfast skipping (Brown *et al.*, 2013), however such a conclusion may not be appropriate as it is unlikely that breakfast comprising 85% energy from fat would regularly be consumed. Combined subjective appetite responses also indicated that a fat-rich breakfast was more effective in reducing appetite following lunch than breakfast omission, although it cannot be directly interpreted that this would lead to a difference in energy intake. Supplementation with whey protein did not appear to significantly augment the appetite response, which contrasts with some previous observations (Solah *et al.*, 2010; Zafar *et al.*, 2013), although neither of these studies included high-fat test meals.

In conclusion, the addition of whey protein to a fat-rich meal does not positively augment the postprandial triglyceride response in healthy young males, despite a moderate increase in acute and second meal insulinaemia, while acute and longer term glycaemic responses were unaffected. Further investigation of postprandial lipaemia in overweight or obese individuals may be warranted, particularly in the context of a mixed-macronutrient test meal.

CHAPTER 5

The effect of timing of whey protein supplementation around breakfast on acute and second-meal metabolic and appetite responses in centrally-obese males

5.1 Introduction

Evidence from the previous two sub-chapters demonstrated the insulinotropic effects of whey protein consumption, however this did not appear to significantly augment postprandial glycaemia in normal-weight, healthy individuals. Whether an effect would be observed in centrally-obese individuals is unclear however. Despite the growing number of studies in normal-weight populations or individuals with T2DM, relatively few studies have been conducted with overweight/obese participants who are free from metabolic disease. This is surprising given that such individuals are likely to be exposed to elevated postprandial glycaemic excursions (Grundy, 2012) and their associated adverse metabolic effects (Gerich, 2006). The accumulation of excessive adipose tissue, particularly in the abdominal region, a condition known as central obesity, is associated with insulin resistance and increased risk of developing T2DM and CVD (Item & Konrad, 2012; O'Neill & O'Driscoll, 2015). Thus,

such individuals may benefit the most from strategies to ameliorate postprandial hyperglycaemia.

In the studies that have been conducted, a significant reduction in glycaemia was observed following consumption of whey protein in obese, but otherwise healthy, female participants (Pal *et al.*, 2010b), and whey produced a lower glucose response compared with a glucose preload in obese males (Bowen *et al.*, 2006b). The relatively large doses of whey used, 45g and 55 g respectively, mean it is unclear whether such an effect would be replicated after administering a more realistic amount of protein, while it is also unclear if participants were centrally obese, as elevated BMI is the only reported obesity criteria.

Previous studies have demonstrated beneficial effects of whey protein consumption on glycaemia with various supplementation approaches. Akhavan *et al.* (2010) reported significant reductions in glycaemia when whey (10-40g) was consumed 30 minutes prior to a pre-set pizza meal in lean participants. Similar benefits have been observed when whey protein is ingested alongside a meal (Petersen *et al.*, 2009). Thus, it is unclear based on the literature whether it is most advantageous to consume whey protein prior to or alongside a meal to promote the greatest acute health benefits. When designs have allowed a preload vs no preload comparison to be made, no significant differences were observed in metabolic (Ma *et al.*, 2009) or subjective appetite (Potier *et al.*, 2010) responses. Nevertheless, Abou-Samra *et al.* (2011) reported that the appetite suppressing effects of other protein sources (casein and pea) after a preload was administered 30 minutes prior to a meal disappeared when consumed immediately prior to the meal.

Many studies have used a preload design, administering whey protein at a set time before a standardised or *ad libitum* meal under all conditions, while fewer studies have administered whey protein alongside or immediately prior to a test meal, as detailed in Chapters 4a and 4b. A large variation in preload timings (15-180 minutes) makes determination of the most efficacious supplementation strategy difficult. To the author's knowledge, the study of Ma *et al.* (2009) is the only one to have directly compared the effect of consuming whey protein before or alongside a meal on subsequent metabolic responses. It should be noted that consuming a preload 30 or more minutes prior to a meal is not analogous to conventional eating habits (Popkin & Duffey, 2010). To have the greatest impact upon public health, interventions should be replicable in a free-living setting, and it may therefore be that tailoring an intervention to enhance its application is of more significance than attaining the greatest changes in certain metabolic responses in the laboratory. In this context, a strategy that appears to have been overlooked is the consumption of whey protein after a meal. Although not representative of a breakfast eating behaviour, such a strategy could potentially mimic the effect of consuming a high-protein dessert.

In addition, there are currently no studies that have investigated the effects of whey protein consumption on postprandial responses beyond a single isolated meal. A second meal effect was not observed in normal-weight participants in Chapters 4a and 4b, however it remains unclear whether whey may have a more prolonged effect in centrally obese participants. Given that a high protein breakfast attenuated postprandial glucose excursions following a standard lunch meal in participants with T2DM (Park *et al.*, 2015), this may be an appropriate area to investigate. This study therefore investigated the effect of timing of whey protein

supplementation on postprandial metabolic and appetite responses in centrally obese males, using timings that reflect habitual eating behaviours.

5.2 Methods

5.2.1 Participants

Participants were recruited as described in section 3.1. Fourteen sedentary centrally-obese male participants, free from metabolic disease, were screened against the inclusion/exclusion criteria listed in Table 3.1. Data from one participant were removed from analysis (see section 5.3), thus participant characteristics are presented in Table 5.1 as $n = 13$. All participants provided written informed consent. This study was registered at clinicaltrials.gov as NCT02658110.

Table 5.1 Participant characteristics.

	All Participants (<i>n</i> = 13)		
Characteristics			
Age (years)	39	±	3
Body mass (kg)	117.9	±	3.7
Stature (cm)	181.3	±	2.8
BMI (kg·m ⁻²)	36.0	±	1.1
Waist circumference (cm)	121.3	±	2.6
Waist/hip ratio	1.00	±	0.01
Fasting variables			
Blood glucose (mmol·l ⁻¹)	5.0	±	0.1
Plasma insulin (pmol·l ⁻¹)	122.0	±	24.8
HOMA-IR	4.6	±	1.0
Plasma triglyceride (mmol·l ⁻¹)	1.91	±	0.17

Fasting values are presented as mean of fasting samples for each main trial.

5.2.2 Experimental design

Participants completed four experimental trials in a randomised, counterbalanced fashion as part of a single-blind, crossover design. Trial order was determined as described in section 3.2. On all visits participants consumed a standardised mixed-macronutrient breakfast meal, followed 180 minutes later by consumption of a standard lunch meal. The timing of additional protein supplementation varied by trial, with participants consuming 20 g whey protein as a preload 15 minutes prior to breakfast (PRE), during the breakfast meal (DUR), or 15 minutes post-breakfast consumption (POST). A control trial was also completed without additional protein supplementation (CON). Prior to each visit, diet and physical activity were standardised as described in section 3.3, and a mixed-macronutrient evening meal (Table 3.2) was provided for participants to consume 12 hours before each trial commenced.

Participants arrived at the laboratory at the same time (~0800 hours) on each occasion following an overnight fast. After insertion of a cannula in an antecubital vein, baseline venous and capillary blood samples were collected (see section 3.6.3), and subjective appetite was assessed using VAS. In the PRE condition, participants subsequently consumed a beverage containing 20 g whey protein, whereas a flavoured water beverage was provided in all other conditions. After 15 minutes, breakfast was consumed in all conditions, accompanied by either a whey protein (DUR) or placebo beverage. At 15 minutes post-breakfast consumption, a further test beverage was provided, with whey protein administered in the POST trial and flavoured water during all other trials. The remainder of the protocol was identical under all experimental conditions, with blood samples and appetite ratings

collected at regular intervals (Figure 5.1). Participants rested in a seated position for 180 minutes following breakfast consumption, after which the same standardised lunch meal was consumed on all occasions. After a further 180 minutes, the cannula was removed and participants could leave the laboratory.

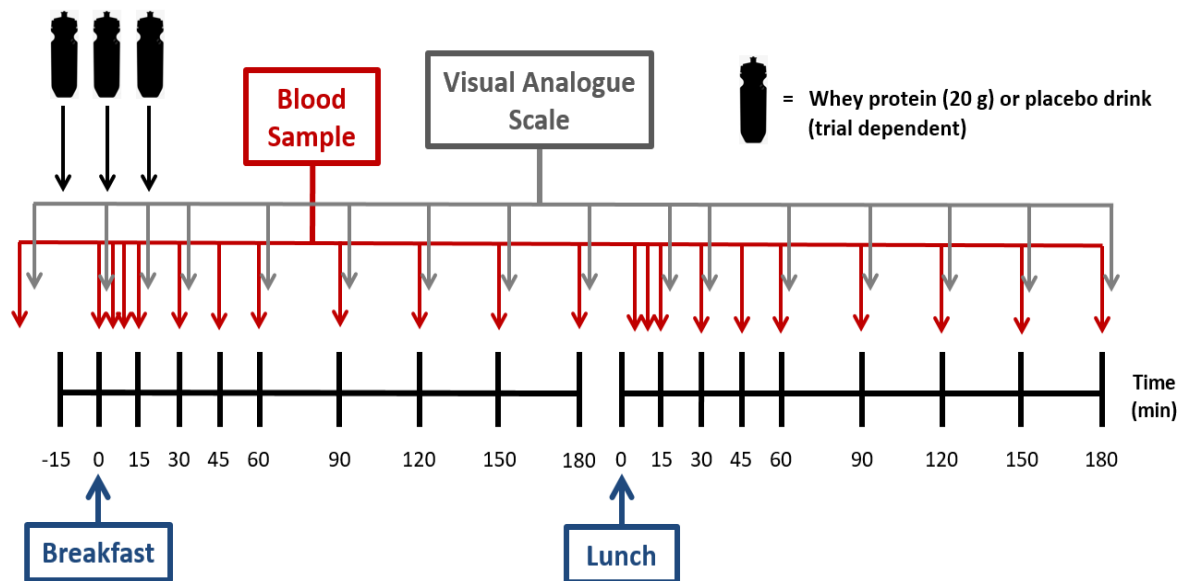


Figure 5.1 Schematic representation of experimental trials.

5.2.3 Test meals

At each test drink time point (15 minutes before, during or 15 minutes after breakfast) either a whey protein or placebo drink was consumed which was condition-dependent. The protein drink consisted of 23g whey protein isolate powder (see section 3.5) mixed with 150 ml water

and 0.5 ml energy-free strawberry flavouring (FlavDrops, Myprotein, UK). The protein content of the supplement was 87% (Appendix F2), providing a 20 g dose of whey protein with a 344 kJ (82 kcal) energy content. The placebo drink consisted of an isovolumetric bolus of similarly flavoured water. All drinks were provided in opaque bottles and no reference was made to which trial was being conducted. An additional 200 ml drinking water was administered after each test beverage to eliminate any after taste.

A standardised breakfast of rolled porridge oats with semi-skimmed milk and honey was provided as under all conditions. This was prepared as described in section 3.4, and provided 1960 kJ (468 kcal) of energy (70% carbohydrate, 17% fat, 13% protein) with a GI of 63. Participants were encouraged to consume this meal within 10 minutes, and 200 ml drinking water was provided alongside the porridge. Porridge was selected to represent a habitually consumed breakfast food amongst the UK population (Reeves *et al.*, 2013) which, in contrast to the test breakfasts administered in Chapters 4a and 4b, provided a more mixed macronutrient breakdown, enhancing the ecological validity of any findings.

A standardised mixed-macronutrient lunch meal was administered in all conditions, consisting of pasta in a cheese and tomato sauce. This was prepared as described in section 3.4, providing 3446 kJ (824 kcal) of energy (50% carbohydrate, 36% fat, 14% protein). Water (500 ml) was also served alongside the lunch meal, and was withheld in the post lunch period.

5.2.4 Blood sampling and analysis

Venous and capillary blood samples were collected at regular intervals (Figure 5.1) while the participant was in a seated position (see section 3.6.3). Additional capillary samples were taken at 5 and 10 minutes post-breakfast and post-lunch in order to increase the resolution of the blood glucose response curve. Samples were processed and analysed for concentrations of glucose, insulin, triglyceride and NEFA as described in section 3.6.4. Due to technical difficulties with the automated analyser (RX Daytona, Randox Laboratories, UK) used for determination of plasma glycerol concentrations, data for this analyte are not presented.

5.2.5 Subjective appetite

Subjective appetite ratings were assessed at regular intervals throughout the protocol (Figure 5.1) using VAS as described in section 3.6.2. Scales assessed perceptions of hunger, fullness, PFC and satisfaction, and a combined appetite score was calculated (section 3.7).

5.2.6 Statistical analysis

Total AUC was calculated using the trapezoidal method (see section 3.7). Data were analysed over both the post-breakfast (0-180 min) and post-lunch (180-360 min) periods. The time point at 180 minutes post-breakfast was used as the baseline value for the post-lunch period. Due to its delayed appearance in plasma, triglyceride data were also assessed over the full 360 minute laboratory protocol. Statistical analysis was conducted as described in section 3.7.1.

5.3 Results

Fasting values did not differ between conditions for all physiological and appetite variables assessed ($p > 0.05$). Analysis revealed that one participant did not adhere to the pre-trial fasting protocol prior to one visit. Data from this participant was subsequently removed from analysis, thus data are presented as $n = 13$.

5.3.1 Post-breakfast responses

Blood glucose responses displayed a significant condition x time interaction effect ($p < 0.001$) in addition to main effects of condition ($p < 0.001$) and time ($p < 0.001$). Glucose peaked at 30 minutes post-breakfast under all conditions (Figure 5.2a), with the magnitude of the peak significantly reduced when whey protein was consumed as a preload or alongside breakfast compared with whey after the meal or no supplementation (PRE: 7.2 ± 0.3 , DUR: 7.8 ± 0.3 vs POST: 8.6 ± 0.3 , CON: 8.6 ± 0.2 mmol·l⁻¹; all $p \leq 0.046$). A tendency for peak to be reduced in PRE compared to DUR was also observed, however this was not statistically significant ($p = 0.076$). Compared to CON, glucose was lower in PRE at 10-45 minutes ($p \leq 0.017$), and in DUR at 30-60 minutes post-breakfast ($p \leq 0.041$). Concentrations returned to baseline levels at 90 minutes post-breakfast under all conditions, with no further between-trial differences observed ($p > 0.05$). Glycaemia across the post-breakfast period was significantly lower following whey preload compared with POST and CON conditions (AUC_{0-180min}: PRE: 982 ± 30 vs POST: 1031 ± 36 and CON: 1065 ± 37 mmol·min⁻¹·l⁻¹; $p \leq 0.042$), but not significantly different from the whey with meal condition (DUR: 1013 ± 32 mmol·min⁻¹·l⁻¹; $p = 0.77$; Figure 5.2b).

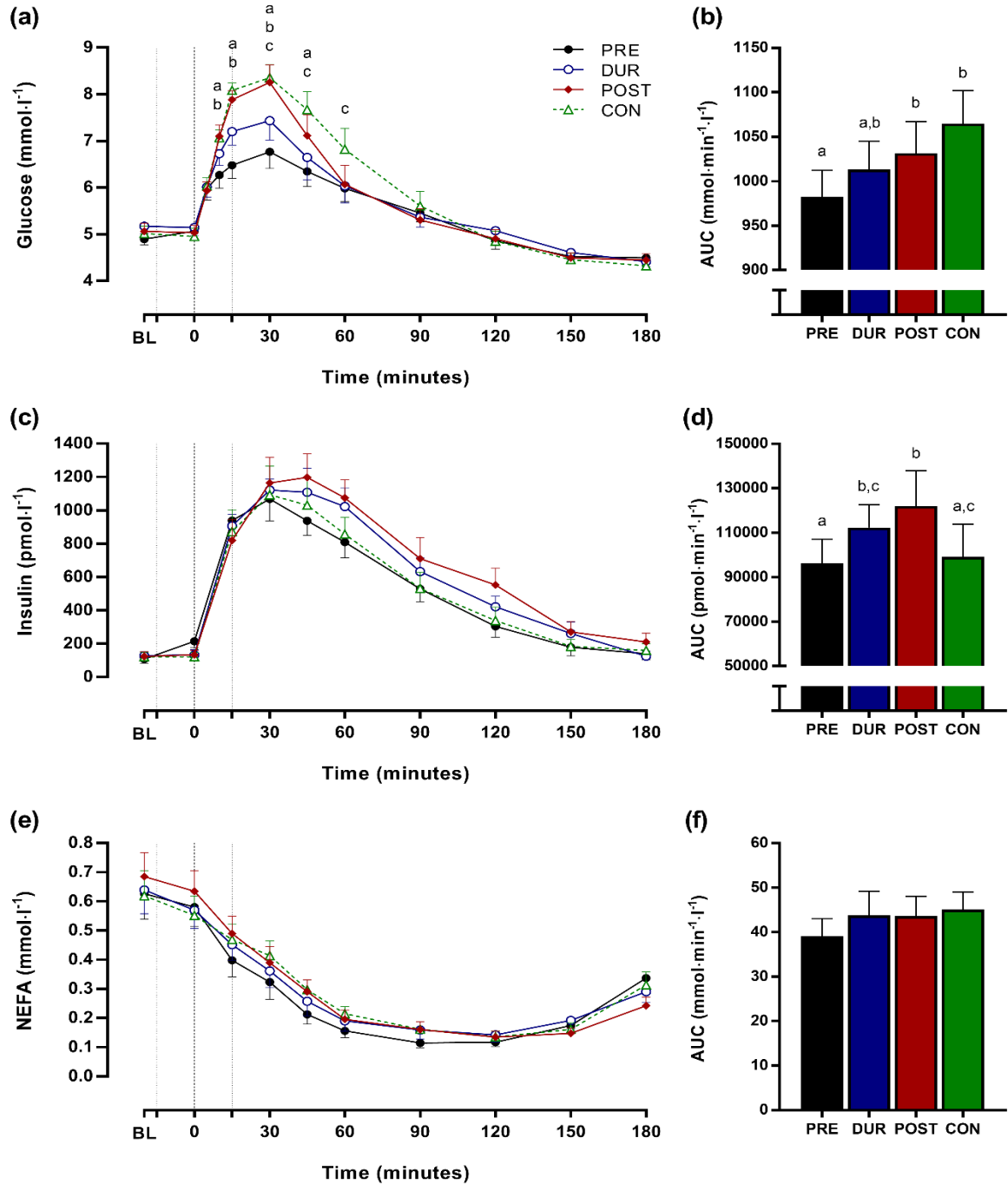


Figure 5.2 Time course changes in glucose (a), insulin (c) and NEFA (e) concentrations during the post-breakfast period (0-180 minutes). Significant differences ($p < 0.05$) between trials at individual time points are defined as follows: a, PRE vs CON; b, PRE vs POST; c, DUR vs CON; black dotted line indicates time of breakfast consumption; grey dotted lines indicate time of whey protein consumption during PRE and POST trials; Area under the curve for glucose (b), insulin (d) and NEFA (f) responses. Bars not sharing a common superscript letter differ significantly from one another ($p < 0.05$). Data are presented as mean \pm SEM. PRE: pre-meal protein trial; DUR: during meal protein trial; POST: post-meal protein trial; CON: no additional protein trial; AUC: area under the curve; NEFA: non-esterified fatty acids.

Postprandial plasma insulin concentrations were influenced by both time ($p < 0.001$) and condition ($p = 0.047$). Concentrations rose following breakfast consumption, remaining significantly elevated above baseline level at 15-120 minutes post-breakfast ($p \leq 0.007$; Figure 5.2c). Post hoc analysis of the main effect of condition indicated that concentrations were lower during PRE than DUR ($p = 0.019$) with a trend for insulin to be lower compared to POST ($p = 0.053$), however concentrations did not differ between PRE and CON ($p > 0.05$). Insulin AUC across this period showed a reduced insulin response when whey was supplemented before breakfast compared with other supplementation times (AUC_{0-180min}: PRE: 96340 ± 10807 vs DUR: 112344 ± 10310 and POST: 121997 ± 15862 pmol·min⁻¹·l⁻¹; $p \leq 0.032$; Figure 5.2d), and was similar to the response following breakfast without additional protein (CON: 99115 ± 14656 pmol·min⁻¹·l⁻¹; $p > 0.05$). When whey was supplemented after the meal, insulinaemia was ~23% greater than control ($p = 0.049$). Insulin sensitivity did not significantly differ between conditions during the post-breakfast period (Matsuda-ISI; PRE: 3.8 ± 0.6 , DUR: 2.9 ± 0.5 , POST: 2.8 ± 0.4 , CON: 3.3 ± 0.5 arbitrary units; $p = 0.161$).

Post-breakfast NEFA concentrations were significantly affected by time ($p < 0.001$) such that levels were immediately suppressed following breakfast consumption under all conditions, and remained significantly below baseline levels from 15-180 minutes post-breakfast ($p \leq 0.006$; Figure 5.2e). There was no effect of condition ($p = 0.611$) and NEFA AUC was similar between conditions across this period ($p = 0.517$; Figure 5.2f).

An effect of time ($p < 0.001$), but not condition ($p = 0.26$) or condition x time interaction ($p = 0.423$), was observed on appetite perceptions following breakfast. Combined appetite

ratings were similarly suppressed under all conditions following breakfast consumption, reaching their nadir at 15-30 minutes post-consumption, the magnitude of which was not different between conditions (PRE: 14 ± 4 , DUR: 15 ± 4 , POST: 17 ± 4 , CON: 20 ± 4 mm; $p = 0.344$; Figure 5.3a). Appetite subsequently increased gradually, reaching similar levels to baseline at 120-180 minutes post-breakfast in all conditions ($p > 0.05$). There were no significant differences in AUC between conditions for combined appetite score (Figure 5.3b) or individual subjective appetite components (Table 5.2) across this period ($p > 0.05$).

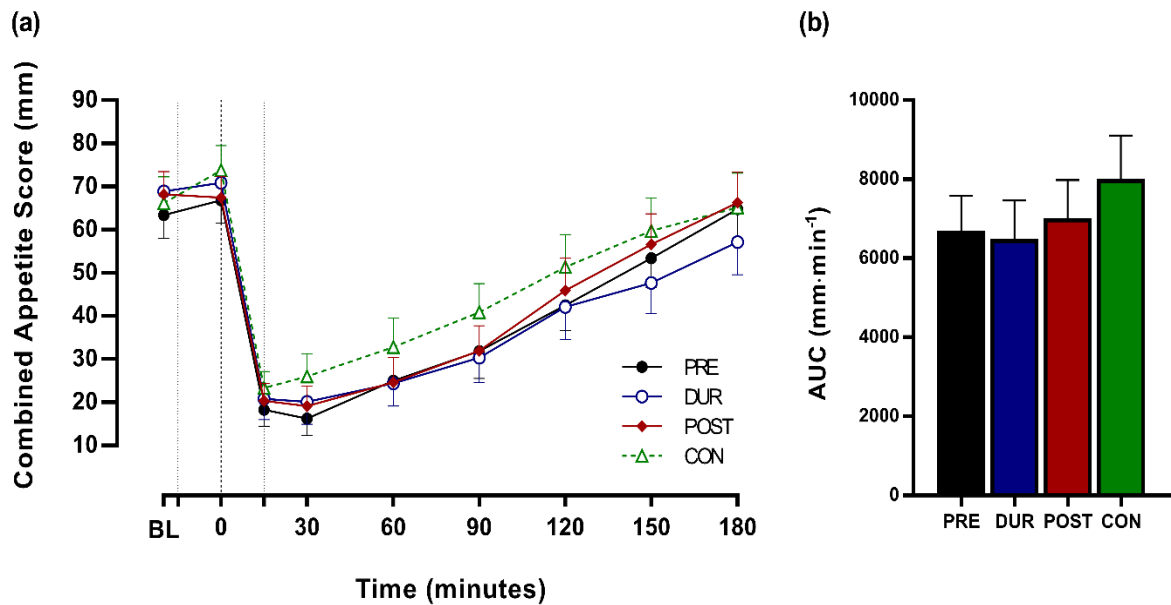


Figure 5.3 Time course changes (a) and area under the curve (b) for combined appetite score during the post-breakfast period (0-180 minutes). Data are presented as mean \pm SEM; black dotted line indicates time of breakfast consumption; grey dotted lines indicate time of whey protein consumption during PRE and POST trials; PRE: pre-meal protein trial; DUR: during meal protein trial; POST: post-meal protein trial; CON: no additional protein trial; AUC: area under the curve.

Table 5.2 Area under the curve for components of subjective appetite during post-breakfast (0-180 minutes) and post-lunch (180-360 minutes) postprandial periods.

		Area under the curve			
		PRE	DUR	POST	CON
Hunger (mm·min ⁻¹)	Post-breakfast	6033 ± 801	6070 ± 911	6461 ± 887	7641 ± 1095
	Post-lunch	6046 ± 734	5651 ± 954	6378 ± 1012	6649 ± 929
Fullness (mm·min ⁻¹)	Post-breakfast	11350 ± 885	11553 ± 964	11107 ± 965	9963 ± 1139
	Post-lunch	11347 ± 824	11980 ± 974	11130 ± 1080	11003 ± 1007
PFC (mm·min ⁻¹)	Post-breakfast	7303 ± 1034	6642 ± 1086	7448 ± 1094	8761 ± 1288
	Post-lunch	7499 ± 1069	6882 ± 1187	7423 ± 1165	7707 ± 1201
Satisfaction (mm·min ⁻¹)	Post-breakfast	11203 ± 929	11194 ± 966	10767 ± 969	10411 ± 982
	Post-lunch	10941 ± 881	11548 ± 1018	11313 ± 979	10767 ± 980

Data presented as mean ± SEM. PRE: pre-meal protein trial; DUR: during meal protein trial; POST: post-meal protein trial; CON: no additional protein trial; PFC: prospective food consumption.

5.3.2 Post-lunch responses

Following consumption of a standardised lunch meal, both glucose and insulin concentrations displayed a significant effect of time ($p < 0.001$), with no condition or interaction effects present ($p > 0.05$). Concentrations peaked similarly across all conditions before returning to pre-lunch levels at 60 and 90 minutes post-lunch for glucose and insulin respectively (Figure 5.4). Post-lunch AUC revealed no differences between conditions in glycaemia ($p = 0.262$) or insulinaemia ($p = 0.271$; Figure 5.4).

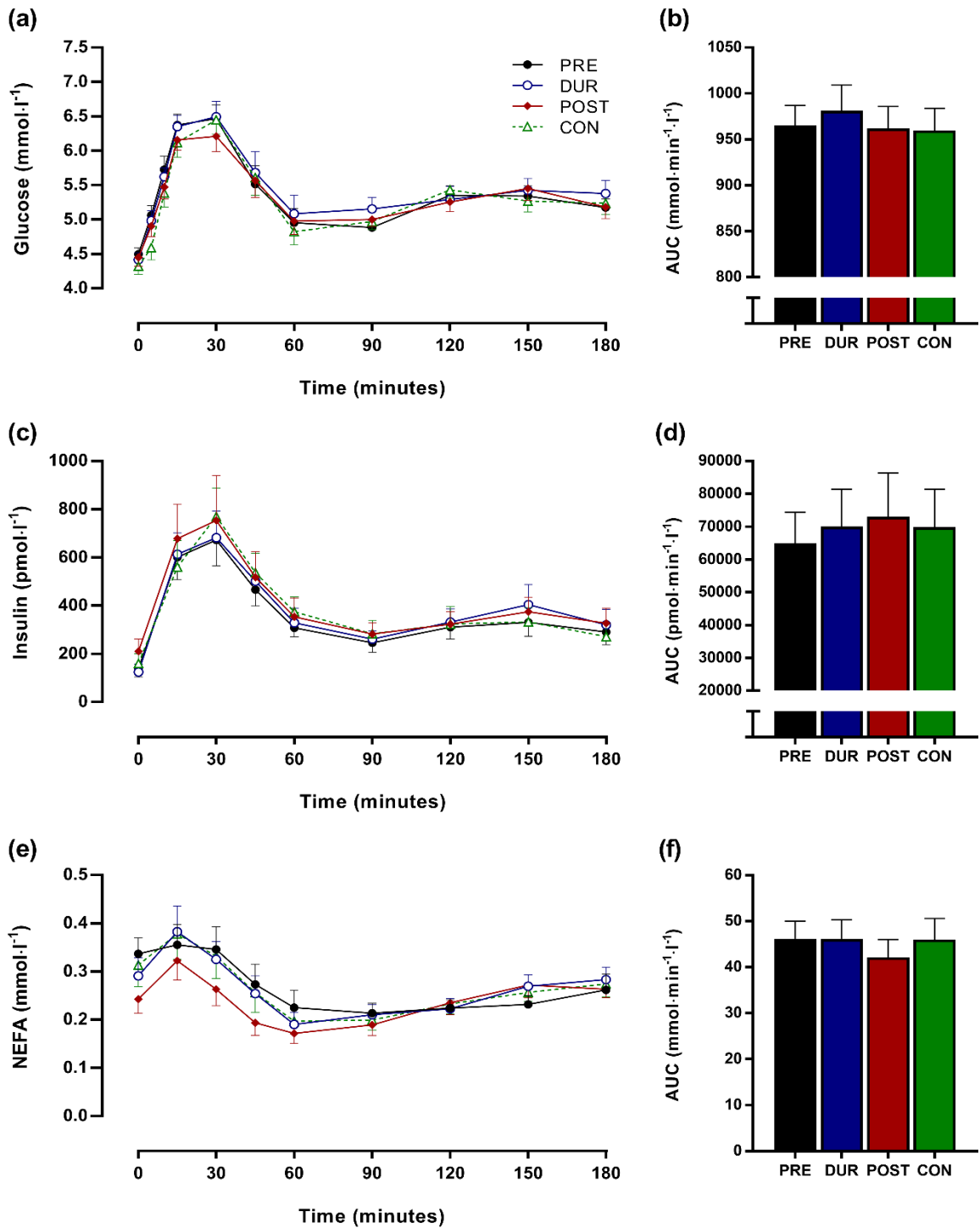


Figure 5.4 Time course changes in glucose (a), insulin (c) and NEFA (e) concentrations during the post-lunch period (180-360 minutes) and area under the curve for glucose (b), insulin (d) and NEFA (f) responses. Data are presented as mean \pm SEM; black dotted line indicates time of breakfast consumption; grey dotted lines indicate time of whey protein consumption during PRE and POST trials; PRE: pre-meal protein trial; DUR: during meal protein trial; POST: post-meal protein trial; CON: no additional protein trial; AUC: area under the curve; NEFA: non-esterified fatty acids.

Post-lunch NEFA concentrations were not influenced by condition ($p = 0.346$), with similar AUC observed between trials ($p = 0.587$; Figure 5.4e). Concentrations were moderately suppressed at 15-60 minutes post-lunch under all conditions, before returning to pre-lunch levels at 90-180 minutes (Figure 5.4f). Appetite was similarly affected by consumption of lunch in all conditions ($p = 0.423$). An effect of time was present ($p < 0.001$), such that there was an immediate reduction in combined appetite score following lunch, before gradually returning to pre-lunch levels at 150-180 minutes post-consumption across all trials (Figure 5.5a). There were no significant differences observed in post-lunch AUC for combined appetite score (Figure 5.5b) or its constituent components of hunger, fullness, PFC and satisfaction (Table 5.2) between conditions ($p > 0.05$).

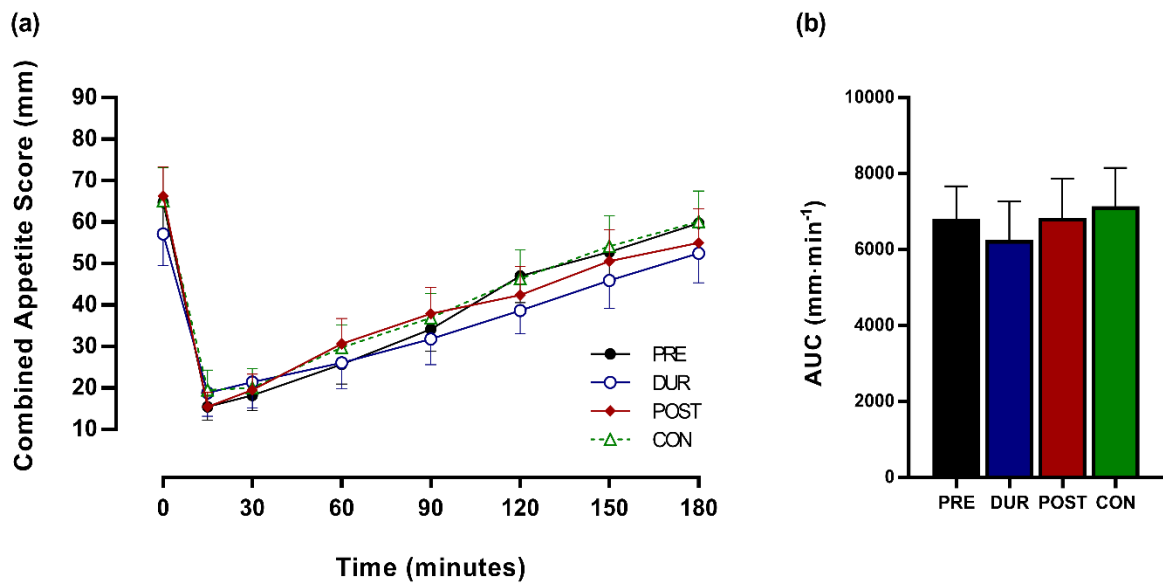


Figure 5.5 Time course changes (a) and area under the curve (b) for combined appetite score during the post-lunch period (180-360 minutes). Data are presented as mean \pm SEM; black dotted line indicates time of breakfast consumption; grey dotted lines indicate time of whey protein consumption during PRE and POST trials; PRE: pre-meal protein trial; DUR: during meal protein trial; POST: post-meal protein trial; CON: no additional protein trial; AUC: area under the curve.

5.3.3 Plasma triglyceride

Triglyceride concentrations increased similarly over the course of the experimental protocol in all conditions. Post hoc analysis of the main effect of time ($p < 0.001$) indicated that levels were elevated above baseline concentration from 60 minutes post-breakfast onwards ($p \leq 0.018$), and continued to rise throughout (Figure 5.6a). There was no effect of condition on triglyceride concentrations ($p = 0.653$), and AUC was similar between conditions for triglyceride response across the full postprandial period ($p = 0.64$; Figure 5.6b).

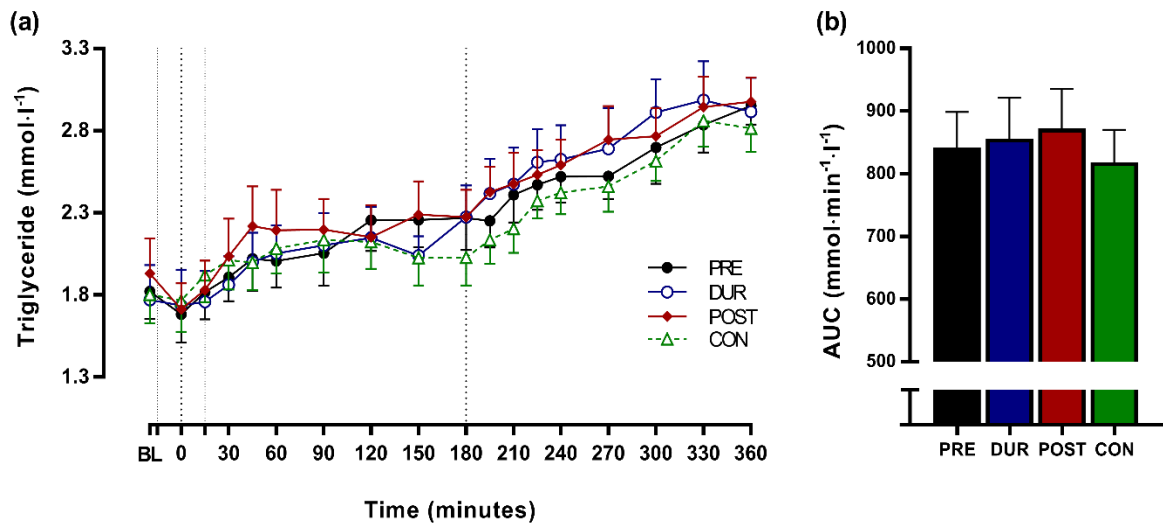


Figure 5.6 Mean \pm SEM temporal changes in plasma triglyceride concentrations throughout the full experimental protocol (0-360 minutes); black dotted line indicates time of breakfast consumption; grey dotted lines indicate time of whey protein consumption during PRE and POST trials; PRE: pre-meal protein trial; DUR: during meal protein trial; POST: post-meal protein trial; CON: no additional protein trial; AUC: area under the curve.

5.4 Discussion

The findings of the current study demonstrate that consumption of whey protein, either before or alongside a mixed-macronutrient breakfast, attenuates peak postprandial glucose excursions in centrally obese, but otherwise healthy, males. Furthermore, consuming whey as a preload appears to confer the greatest beneficial effect, manifested by a reduction in glycaemia over 3 hours compared to the same breakfast devoid of additional protein, without augmenting the postprandial insulin response. In agreement with findings in normal-weight participants described in Chapters 4a and 4b, the addition of 20 g whey protein to a breakfast meal did not significantly affect postprandial lipaemic responses or subjective perceptions of appetite, acutely or following a subsequent lunch meal.

Both peak glucose and mean postprandial glycaemia following meal consumption are significant outcomes in non-diabetic populations presenting with metabolic risk factors such as central obesity. Postprandial hyperglycaemia is a greater risk factor for CVD than increased fasting glucose in non-diabetics (Bianchi *et al.*, 2008; Gerich, 2003), where its contribution to overall glycaemia is particularly marked. Furthermore, postprandial glycaemic excursions have been established as the main causative factor in glycaemic variability in non-insulin treated individuals with IGT (Monnier *et al.*, 2012), and such excursions manifest adverse metabolic effects via activation of oxidative stress and endothelial dysfunction (Ceriello *et al.*, 2008). Thus, evidence supports recommending reductions in postprandial hyperglycaemia as a relevant clinical goal in delaying or preventing the onset of T2DM (Monnier & Colette, 2015).

The reduction in peak glucose observed following a whey preload in the present study ($1.4 \text{ mmol}\cdot\text{l}^{-1}$) is comparable with those demonstrated in previous studies in both healthy and diabetic populations. Akhavan *et al.* (2010) reported a mean peak decrement of $1.8 \text{ mmol}\cdot\text{l}^{-1}$ following a mixed-macronutrient meal preceded by 20 g whey protein, albeit with a longer duration between preload and meal (30 minutes). In prediabetic individuals and those with T2DM, Clifton *et al.* (2014a) observed a $2.1 \text{ mmol}\cdot\text{l}^{-1}$ reduction in peak blood glucose when a high GI breakfast was consumed 15 minutes after 17 g whey protein. The addition of 5 g guar fibre to this preload, known to delay gastric emptying (Sah *et al.*, 2016) may explain the greater effect. Significantly reduced glucose peaks were also observed when whey protein was supplemented at the same time as breakfast consumption in the present study, however the magnitude of this reduction compared to control ($\sim 9\%$) was lower than during the preload condition ($\sim 14\%$), with the difference between these conditions approaching significance. This is suggestive of a greater beneficial effect when whey is administered at least 15 minutes prior to a meal. Indeed, the finding that total AUC was significantly reduced over the full postprandial period in the preload trial, compared to both control ($\sim 8\%$) and post-meal supplementation ($\sim 5\%$), whereas glycaemia did not differ significantly from other conditions in the with-meal trial, appears to confirm this.

Several mechanisms are likely to have interacted to bring about such an effect. The insulinotropic effect of whey protein has previously been established (Gunnerud *et al.*, 2013; Nilsson *et al.*, 2007), and may be amplified by a possible inhibitory effect of whey on DPP-IV, which acts to degrade the incretins (Jakubowicz & Froy, 2013). Plasma insulin was significantly greater when supplemented after breakfast in the current study, however this did not lead to a significant lowering of blood glucose compared to control. When whey was

administered as a preload however, glycaemia was significantly lower than control in the absence of a differential insulin response, indicative of an insulin-independent effect. An effect on gastric emptying is a likely mediator for an insulin-independent effect (Akhavan *et al.*, 2010). It is well established that the rate of gastric emptying is a major determinant of postprandial blood glucose excursions, particularly in the early postprandial phase (Marathe *et al.*, 2013; Mignone *et al.*, 2015). Consumption of a whey protein preload has previously been reported to slow gastric emptying (Akhavan *et al.*, 2014) and, although this was not measured in the current study, it could be speculated that such an effect was responsible for the observed reduction in glycaemia. In addition, the fact that glycaemia was greater when whey was consumed 15 minutes after the meal compared with the preload trial, despite significantly greater insulinaemia, suggests that consumption was too late to significantly affect the rapid absorption of exogenous glucose (Frayn, 2010), which peaked in the blood just 15 minutes later.

The efficacy of both fat (Gentilcore *et al.*, 2006) and protein (Hall *et al.*, 2003) preloads in slowing gastric emptying has been shown previously, however the greater energy density of fat may limit its application. Nutrient intake initiates the release of the incretin hormones, while GLP-1 also acts to slow gastric emptying and suppress glucagon release (Mignone *et al.*, 2015). In addition, other gut peptides such as CCK and PYY, released due to the presence of nutrients in the gastrointestinal tract, act to slow gastric emptying (Marathe *et al.*, 2013). The fact that pre-meal protein reduced post-meal glycaemia in the present study, whereas the same amount of protein with the meal did not have a significant effect, contrasts with the findings of Ma *et al.* (2009). In this study in a T2DM population, protein slowed gastric emptying to a greater extent after a preload, however glycaemia was reduced significantly

under both conditions without an effect of preload. The considerably larger dose of protein used (55 g) and high GI nature of the test meal may explain this difference, as a 3-fold increase in insulin concentration was observed 90 minutes after consumption of the test meal in supplementation conditions.

The ability of whey protein to improve glycaemia without a concomitant increase in plasma insulin has been reported previously (Akhavan *et al.*, 2010), and may be indicative of transient changes in insulin sensitivity due to whey protein ingestion. Enhanced insulin sensitivity has been shown following chronic whey protein supplementation in both rats (Belobrajdic *et al.*, 2004) and humans (Pal *et al.*, 2010a). However this contrasts with the reported promotion of insulin resistance with increased amino acid availability (Tremblay *et al.*, 2005) following acute whey protein ingestion (Smith *et al.*, 2015). The composite index of insulin sensitivity (Matsuda-ISI) did not differ significantly between conditions in the current study, indicating that insulin-independent mechanisms such as delayed gastric emptying may have had a greater influence on postprandial glycaemia.

The effects of whey protein ingestion, independent of timing of consumption, were limited to glucose and insulin responses in the current study. Postprandial lipaemia was not affected by co-ingestion of whey protein with breakfast, which differs from the findings of Pal *et al.* (2010b). This group observed a 21% reduction in triglyceride appearance in plasma following supplementation of 45 g whey, compared with glucose, alongside a mixed-macronutrient meal in obese females. Such a difference is perhaps unsurprising due to the considerably lower fat content of the breakfast administered in the present study (9 g) compared to the

aforementioned study (32 g), whilst the amount of supplemental whey administered by Pal *et al.* (2010b) was over 2-fold greater.

Subjective appetite responses were also similar across conditions in the present study, regardless of the addition of whey or its timing. Although post-breakfast appetite appeared to be elevated in the control condition, this difference was not significant and may be a consequence of the fact that this study was powered to detect differences in the primary outcome of glycaemia. The lack of a timing effect supports the findings of a previous study where the consumption of carbohydrate, fat and protein preloads had similar effects on both food intake and subjective appetite whether ingested immediately or 1 hour before a lunch meal (Potier *et al.*, 2010). In contrast, Abou-Samra *et al.* (2011) reported that food intake was reduced following casein and pea preloads (30 minutes prior to *ad libitum* meal) compared to a water control, however this effect was not present when preloads were consumed immediately prior to the meal. Such an effect may be limited to other proteins however, as whey did not significantly affect food intake or appetite in this comparison. A positive effect of whey protein on subjective appetite responses has previously been shown in overweight males after a 55 g preload (Bowen *et al.*, 2006b), however such a comparison with glucose does not easily compare with the response following the addition of 20 g whey to a mixed-macronutrient breakfast meal consisting of habitually consumed ingredients. A mixed effect of whey on subjective appetite in healthy individuals has previously been shown. Several studies have reported reductions in appetite (Hall *et al.*, 2003; Solah *et al.*, 2010; Zafar *et al.*, 2013), although the mechanisms remain to be elucidated. Others have failed to show an effect (Abou-Samra *et al.*, 2011; Astbury *et al.*, 2010; Chungchunlam *et al.*, 2015), however these responses did not necessarily correlate with energy intake.

Whey protein at breakfast did not affect post-lunch responses across all outcomes assessed in the present study. This appears to confirm the findings of Chapter 4a in normal-weight individuals, and suggests that any effects of whey protein on postprandial glycaemia are transient, and may provide rationale for researchers to investigate the supplementation of whey protein at multiple sequential meals.

This study aimed to investigate the effect of timing of whey protein supplementation on postprandial metabolic and appetite responses. It is unclear if the beneficial effects on glycaemia would have been greater with a longer duration between preload and test meal, however the timings used in the present study were designed to reflect an approach that does not deviate markedly from typical eating habits. As such, consuming a small nutrient load 15 minutes before a meal is analogous to consuming a starter prior to a main meal, and may therefore be more easily replicated in the free-living setting.

In summary, consumption of whey protein alongside a mixed-macronutrient meal attenuated postprandial glucose excursions in centrally-obese males. In addition, consumption of whey as a preload had a similar effect on peak glucose, also attenuating glycaemia over the subsequent 3 hours, with a simultaneously reduced insulin response. Reductions in postprandial glycaemia may be beneficial in preventing or delaying the progression from NGT to IGT or T2DM. The addition of whey protein, independent of timing, did not affect postprandial responses to a subsequent lunch meal.

CHAPTER 6

Metabolic and appetite responses to a whey protein preload following prior low-moderate intensity exercise in sedentary centrally-obese males

6.1 Introduction

Obesity, particularly of the abdominal region, is associated with dysregulation of a number of metabolic processes including glucose and lipid metabolism, and thus increased risk of developing T2DM and CVD (Misra & Khurana, 2008). Lifestyle intervention is an effective strategy in the prevention of chronic diseases (Hays *et al.*, 2008) and dietary interventions that aim to reduce body mass via energy restriction are common (Andersen & Fernandez, 2013). However, adherence to such interventions and maintenance of improvements are often areas of concern (Alhassan *et al.*, 2008; Anderson *et al.*, 2001). Thus, additional strategies to reduce the risks associated with central obesity are important, including modulating postprandial responses to reduce the adverse metabolic effects associated with elevated postprandial glycaemia and lipaemia (Gerich, 2006; Kolovou *et al.*, 2011).

In chapter 5 it was found that consumption of whey protein 15 minutes prior to a meal can attenuate postprandial glycaemia in obese males. Furthermore, this effect was observed using a smaller supplemental whey protein dose (20 g vs 45-55 g) than previous studies showing the same effect in obese, but otherwise healthy participants (Bowen *et al.*, 2006b; Pal *et al.*, 2010b). Subjective appetite was unaffected by the same intervention, however as subsequent intake was not measured it is unclear if energy intake is influenced at a later meal. Whey may also influence postprandial energy expenditure through an increase in diet-induced thermogenesis (Acheson *et al.*, 2011; Hursel *et al.*, 2010). Evidence therefore indicates the potential of whey protein supplementation to positively influence post-meal responses.

Physical activity and exercise are also important aspects of chronic disease prevention. The UK Government recommends achieving at least 30 minutes of moderate or greater intensity physical activity (such as brisk walking) on five or more days a week (National Institute for Health and Care Excellence, 2014), and a joint position stand of the American College of Sports Medicine and the American Diabetes Association affirms this goal for reduction of T2DM risk (Colberg *et al.*, 2010). Evidence suggests that greater amounts of physical activity are required to influence central obesity and maintenance of fat loss (Slentz *et al.*, 2009), however such targets may not be sustainable in the free-living setting for the majority of obese individuals who currently perform very little physical activity (Bray *et al.*, 2016). Moreover, exercise training does not result in weight loss for the majority of obese individuals (Hays *et al.*, 2008), and compliance is often poor (Johnson & Jebb, 2009). In this context, and given that the effects of exercise on cardiometabolic risk factors are often found to be transient (Boulé *et al.*, 2005; Colberg *et al.*, 2010), investigating the acute effects of a

single bout of exercise on postprandial metabolism may be relevant in populations that are unlikely to undertake frequent training.

Acute endurance-type exercise is a potent regulator of postprandial lipid metabolism, with studies consistently reporting a reduction in postprandial triglyceride response following a high-fat meal after prior exercise (Freese *et al.*, 2014). Evidence also suggests that acute exercise does not increase hunger or food intake in normal-weight (Martins *et al.*, 2008) and overweight/obese individuals (Martins *et al.*, 2015). This underlines the potential for an acute bout of exercise to induce an energy deficit, however laboratory-based studies have revealed a large degree of inter-individual variation in post exercise compensatory eating in lean (Finlayson *et al.*, 2009) and obese (Hopkins *et al.*, 2013) individuals. A single bout of moderate-intensity aerobic exercise is sufficient to increase insulin sensitivity in habitually inactive obese adults (Nelson & Horowitz, 2014). The effect on acute post-exercise glycaemic responses is equivocal however, with reported improvements (Hasson *et al.*, 2010), no change (Gonzalez *et al.*, 2013) or decrements (Rose *et al.*, 2001) in glucose tolerance. Glucose uptake tends to be elevated for several hours post-exercise (Colberg *et al.*, 2010), however the rate of exogenous glucose appearance from a post-exercise meal may also be augmented. The fitness and prior nutritional state of the participant are likely to influence post-exercise metabolism, however the effect of altering post-exercise nutritional intake is unclear.

The vast majority of studies concerning post-exercise whey protein supplementation have been conducted following resistance-type exercise, with beneficial effects on muscle protein synthesis (Witard *et al.*, 2014) and lean mass maintenance (Hayes & Cribb, 2008; Naclerio

& Larumbe-Zabala, 2015) previously described. However, the influence of whey protein supplementation following aerobic exercise on subsequent metabolic and appetite responses has received little attention. Reduced *ad libitum* energy intake has been observed 60 minutes after milk (Rumbold *et al.*, 2015) or whey protein (Clayton *et al.*, 2014) consumption following prior moderate-intensity cycling exercise in recreationally active participants. Whether postprandial metabolic and appetite responses would be influenced by post-exercise whey protein consumption in habitually inactive obese individuals remains unclear. Given that a single bout of exercise may produce divergent responses in subsequent glucose tolerance (Gonzalez, 2014), and that whey has been observed to influence postprandial glycaemia and insulinaemia within this thesis, the impact of post-exercise whey consumption on postprandial metabolic responses may be of significance.

Acute endurance-type exercise and whey protein supplementation have previously been reported to affect acute metabolic and appetite responses to a subsequent meal independently, however it is unclear whether additional beneficial effects are present when these strategies are combined. This study therefore aimed to investigate the effect of fasted moderate-intensity exercise and subsequent whey protein supplementation on postprandial metabolic and appetite responses.

6.2 Methods

6.2.1 Participants

Twelve sedentary centrally-obese male participants, free from metabolic disease, took part in the study (see Table 6.1 for participant characteristics). Participant recruitment methods are detailed in section 3.1, and participants were screened against the inclusion/exclusion criteria listed in Table 3.1. All participants provided written informed consent. This study was registered at clinicaltrials.gov as NCT02714309.

Table 6.1 Participant characteristics.

	All Participants (<i>n</i> = 12)		
Characteristics			
Age (years)	41	±	9
Body mass (kg)	121.9	±	10.9
Stature (cm)	179.5	±	5.9
BMI (kg·m ⁻²)	37.8	±	2.1
Waist circumference (cm)	123.4	±	9.9
Waist/hip ratio	1.01	±	0.07
VO _{2peak} (ml·kg ⁻¹ ·min ⁻¹)	25.5	±	3.9
Fasting variables			
Blood glucose (mmol·l ⁻¹)	5.2	±	0.6
Plasma insulin (pmol·l ⁻¹)	122.6	±	56.7
HOMA-IR	4.7	±	2.4
Plasma triglyceride (mmol·l ⁻¹)	1.50	±	0.63

Fasting values are presented as mean of fasting samples for each main trial.

6.2.2 Experimental design

Participants completed three trials in a randomised, counterbalanced fashion, separated by at least 7 days as part of a single-blind, crossover design. Randomisation of trial order was determined as described in section 3.2. Pre-trial diet and physical activity was standardised as described in section 3.3. A 30 minute bout of steady-state brisk treadmill walking was completed on two separate occasions, followed by consumption of a whey protein (EX+PRO) or placebo (EX) preload beverage. On a third occasion, participants remained in a sedentary position for the same duration followed by consumption of the placebo beverage (CON). A standardised, carbohydrate-rich, breakfast meal was provided 15 minutes after consumption of test preloads (whey protein or placebo) under all conditions. Participants remained sedentary for a further 240 minutes, followed by consumption of an *ad libitum* mixed-macronutrient lunch meal.

The efficacy of supplemental whey protein in reducing acute postprandial glycaemia was observed to be greater when whey was given as a preload, rather than alongside, the same standardised breakfast in Chapter 5, therefore this strategy has been replicated in the current study. In previous chapters, a standardised lunch meal has been administered to assess second meal metabolic and appetite responses following whey protein supplementation at breakfast. Having confirmed that prior whey does not influence subsequent meal responses, an *ad libitum* lunch meal was administered in the current study, enabling assessment of between-condition differences in energy intake at this time point.

6.2.3 Preliminary exercise test

Prior to experimental visits, participants completed a treadmill walking test to determine their prescribed walking speed during the main trials. Due to the sedentary nature of participants, a submaximal test was conducted, where linear relationships between walking speed and oxygen consumption, and between heart rate and oxygen consumption, were established. This enabled estimation of $\dot{V}O_{2\text{peak}}$ for each participant, and subsequently calculation of walking speed of the desired relative intensity. Participants warmed up for 3 minutes at a self-selected comfortable walking speed, enabling habituation with the motorised laboratory treadmill (Pulsar 3p, h/p/cosmos, Germany). The test began with the participant walking at 3-4 km·h⁻¹, with four steady-state walking stages completed in total. Each stage lasted for 3 minutes, and the speed of the treadmill was increased by 0.5 or 1 km·h⁻¹ at the end of each stage, unless heart rate exceeded 85% age-predicted maximum (220 – age) or the participant reported any undue side-effects, at which point the test was terminated. The increase in speed was determined according to the discretion of the researcher, and based on the rating of perceived exertion (RPE) which the participant indicated in the final 30 seconds of each stage using the scale of Borg (1990) (Appendix H).

Throughout the test, expired gas was sampled using a breath by breath gas analyser (Oxycon Pro, CareFusion, USA) and heart rate was recorded using short-range telemetry (Polar RS400, Polar Electro, Finland). Data were averaged over 30 second intervals to plot the relationship between oxygen consumption and heart rate, which was extrapolated to age-predicted maximum heart rate in order to estimate $\dot{V}O_{2\text{peak}}$ for each participant. The walking speed eliciting an intensity of 50% $\dot{V}O_{2\text{peak}}$ was determined from the relationship between

oxygen consumption and walking speed, and selected as the prescribed walking speed for exercise bouts during main trials.

6.2.4 Main trial procedures

Participants arrived at the laboratory following an overnight fast, and body mass was recorded to the nearest 0.05 kg using a digital scale (Seca 704, Seca Ltd, Germany) after voiding. Following insertion of a cannula into an antecubital vein, venous and capillary blood samples were taken (see section 3.6.3), and baseline measures of subjective appetite and expired gas were collected. During CON participants remained rested and during EX+PRO and EX a 30 minute bout of steady-state brisk walking was performed on a motorised treadmill. Participants began walking at their predetermined speed, which was subsequently adjusted during the first visit if a significant deviation from the desired value of oxygen consumption was observed during expired gas analysis. Any adjustments were recorded and replicated in the second exercise trial. Heart rate was monitored throughout, and RPE was recorded at 5 minute intervals. The mode, intensity and duration of exercise was designed to be achievable for habitually sedentary individuals, while also conforming to current recommendations for prescribed levels of daily physical activity for health (National Institute for Health and Care Excellence, 2014).

Within 5 minutes of exercise completion, participants consumed a test beverage containing whey protein during EX+PRO, and a placebo beverage during EX. During CON, a placebo beverage was consumed at the corresponding time point. The remainder of the trial procedure was identical under all conditions. A standardised breakfast was consumed 15 minutes after

test beverage ingestion, and participants subsequently remained in a sedentary position with access to reading material or a laptop computer. After 240 minutes had elapsed, a lunch meal was provided to assess energy intake. Upon completion, the cannula was removed and participants were free to leave the laboratory. Blood, expired gas and VAS were sampled at regular intervals throughout (see Figure 6.1).

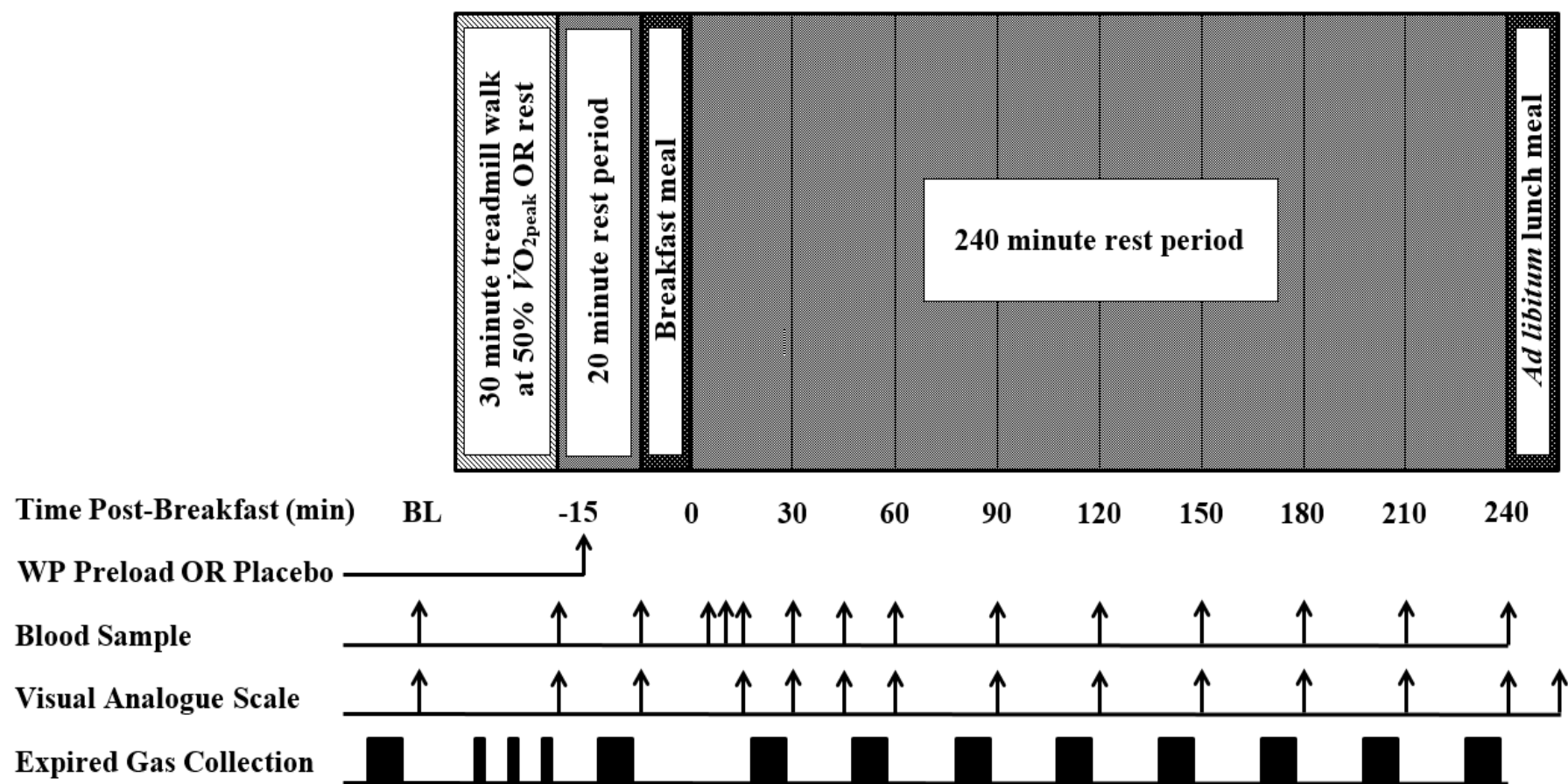


Figure 6.1 Schematic representation of experimental trials. WP, whey protein.

6.2.5 Test meals

During EX+PRO, the test beverage consisted of 23 g whey protein isolate powder (see section 3.5) combined with 150 ml water and 0.5 ml energy-free strawberry flavouring (FlavDrops, Myprotein, UK). The protein content of the supplement was 87% (Appendix F2), providing a 20 g dose of whey with a 344 kJ (82 kcal) energy content. During EX and CON an isovolumetric bolus of similarly flavoured water was consumed as a placebo. All test drinks were provided in opaque bottles. An additional 200 ml drinking water was administered after each test beverage to eliminate any after taste.

A standardised portion of rolled porridge oats with semi-skimmed milk and honey was provided as breakfast under all conditions. This was prepared as described in section 3.4, and provided 1960 kJ (468 kcal) of energy (70% carbohydrate, 17% fat, 13% protein). Participants were encouraged to consume this meal within 10 minutes, and 250 ml drinking water was provided alongside the porridge. A timer was started upon completion of this meal.

A homogenous pasta meal was provided *ad libitum* at lunch to record energy intake. This consisted of dried pasta, a tomato-based sauce, cheddar cheese and olive oil (Tesco Stores Ltd, UK) as used previously (Gonzalez *et al.*, 2015; Gonzalez *et al.*, 2013) providing 53%, 14% and 33% energy from carbohydrate, protein and fat respectively. Participants were initially provided with a 400 g (2845 kJ, 680 kcal) portion of the pasta and were instructed to eat until they felt ‘comfortably full’ on each occasion. The serving bowl was topped up with fresh pasta prior to completion, thus removing the effect of bowl clearance as a stimulus

for food intake termination. All cooked pasta (served or unserved) was weighed immediately before and after consumption to determine energy intake.

6.2.6 Indirect calorimetry

Expired gas was sampled at regular intervals throughout resting and exercise portions of the protocol (Figure 6.1) using an online gas analyser (Oxycon Pro, CareFusion, USA) which was calibrated prior to each use with 3 litre syringe and gasses of known concentrations. Participants wore a facemask (model 7940, Hans-Rudolph, USA) which was connected to the analyser, and remained in a seated position throughout resting samples. Samples were collected at baseline, immediately after test beverage, 20 minutes post breakfast, and every subsequent 30 minutes until the end of the protocol. Expired gas was collected for 10 minute periods, with data from the first and last minute of each period discarded. During treadmill walking, expired gas was collected for 5 minute periods at 5, 15 and 25 minutes into the bout, with the first and last minutes discarded.

Values of oxygen consumption and carbon dioxide production were used to calculate rates of substrate oxidation using stoichiometric equations as follows:

$$\text{Carbohydrate oxidation at rest (g}\cdot\text{min}^{-1}\text{)} = 4.55 \dot{V}\text{CO}_2 - 3.21 \dot{V}\text{O}_2$$

$$\text{Carbohydrate oxidation during exercise (g}\cdot\text{min}^{-1}\text{)} = 4.210 \dot{V}\text{CO}_2 - 2.962 \dot{V}\text{O}_2$$

$$\text{Lipid oxidation (g}\cdot\text{min}^{-1}\text{)} = 1.67 \dot{V}\text{CO}_2 - 1.67 \dot{V}\text{O}_2$$

The methods of Frayn (1983) were used to calculate lipid oxidation and carbohydrate oxygen at rest, which assumes exclusive use of glucose for carbohydrate metabolism. The method of Jeukendrup and Wallis (2005) was used to calculate carbohydrate oxidation during exercise which adjusts for the increase in glycogen contribution to carbohydrate metabolism during moderate intensity exercise. Energy expenditure was calculated based on lipids, glucose and glycogen providing 37.7, 15.5 and 17.2 kJ·g⁻¹ (9.0, 3.7 and 4.1 kcal·g⁻¹), respectively. Urine was collected throughout each visit to determine urinary nitrogen excretion as a marker of protein oxidation. Due to technical difficulties with the automated analyser (RX Daytona, Randox Laboratories, UK), these determinations were not achieved. Protein oxidation contributes least to total energy expenditure, and intake at the level supplemented in the current study (20 g) does not increase protein oxidation above basal levels (Moore *et al.*, 2009; Witard *et al.*, 2014). Therefore, calculations were based on the assumption of negligible contribution of protein oxidation, which reflects the method used in the vast majority of studies utilising indirect calorimetry.

6.2.7 Blood sampling and analysis

Venous and capillary blood samples were collected at regular intervals (baseline, post-exercise/control [-15], 0, 15, 30, 45, 60, 90, 120, 150, 180, 210 and 240 minutes post-breakfast (Figure 6.1)) while the participant was in a seated position, as described in section 3.6.3. Additional capillary samples were taken at 5 and 10 minutes post-breakfast in order to increase the resolution of the blood glucose response curve. Samples were processed and analysed for concentrations of glucose, insulin, triglyceride and glycerol as described in

section 3.6.4. Due to unforeseen complications with the analysis of plasma samples for NEFA concentration, levels of this analyte are not reported in the current study.

6.2.8 Subjective appetite

Subjective appetite ratings were assessed using VAS (section 3.6.2) and a combined appetite score was subsequently calculated, as described in section 3.7. Ratings for hunger, fullness, PFC and satisfaction were collected at corresponding time points to venous blood samples (Figure 6.1), with a final VAS completed following termination of the lunch meal.

6.2.9 Statistical analysis

Total AUC was calculated for subjective appetite and blood analyte data using the trapezoidal method as described in section 3.7. Baseline comparisons between trials, AUC for all variables, measures of energy balance and substrate metabolism were assessed using one-way repeated measures ANOVA. Two-way repeated measures ANOVA (condition x time) was used to test for differences between conditions over time as described in section 3.7.1.

6.3 Results

6.3.1 Exercise responses

Exercise intensity, oxygen consumption, heart rate, perceived exertion, substrate oxidation and energy expenditure were similar between bouts of brisk treadmill walking during EX+PRO and EX conditions (all $p > 0.05$) (Table 6.2).

Table 6.2 Physiological responses during 30 minutes of treadmill walking.

	EX+PRO	EX
Walking Speed (km·h ⁻¹)	5.1 ± 0.5	5.1 ± 0.5
% $\dot{V}O_{2peak}$	51.5 ± 1.4	49.7 ± 1.0
$\dot{V}O_2$ (ml·kg ⁻¹ ·min ⁻¹)	13.0 ± 0.5	12.6 ± 0.4
Heart rate (b·min ⁻¹)	103 ± 12	103 ± 11
RPE	11 ± 2	11 ± 2
CHO oxidation (g)	31.4 ± 2.6	30.1 ± 2.7
Lipid oxidation (g)	10.7 ± 1.3	10.7 ± 1.3
Energy expenditure (kJ)	938 ± 48	914 ± 43

RPE, Rating of perceived exertion; CHO, carbohydrate; EX+PRO, exercise with whey protein preload trial; EX, exercise trial.

6.3.2 Blood glucose and plasma insulin

There were no between-trial differences in blood glucose concentration at baseline ($p > 0.05$). Glucose displayed a significant condition x time interaction effect ($p < 0.001$), time effect ($p < 0.001$) and main effect of condition ($p = 0.009$; Figure 6.2a). The post-breakfast increase

in glucose was reduced after whey protein supplementation compared with placebo trials at 15-30 minutes post-breakfast, and a significantly reduced peak was observed in this condition (EX+PRO: 7.6 ± 0.4 vs EX: 8.4 ± 0.3 , CON: 8.3 ± 0.3 mmol·l⁻¹, $p \leq 0.04$). Glucose was significantly lower during CON than EX+PRO and EX at 90 minutes, and lower than EX+PRO at 120 minutes post-breakfast (all $p < 0.05$). Values declined significantly below baseline levels after 180 minutes in all conditions. Acute (0-60 minutes) postprandial glucose AUC was lower after exercise followed by whey protein consumption than exercise alone (Table 6.3; $p = 0.011$), but not significantly different from CON ($p = 0.12$) after correcting for multiple comparisons. Over the full postprandial period (0-240 minutes), glycaemia was greater during EX compared with CON ($p = 0.002$) but not significantly higher than EX+PRO (Table 6.3; $p = 0.241$).

Plasma insulin concentrations were not different between trials at baseline or immediately post exercise, but were significantly greater following the whey protein beverage (immediately prior to breakfast) compared with CON (EX+PRO: 249 ± 32 vs CON: 118 ± 13 pmol·l⁻¹, $p < 0.001$), but not EX (151 ± 44 pmol·l⁻¹, $p = 0.379$). Insulin displayed a significant interaction of condition and time ($p = 0.006$), and main effects for time ($p < 0.001$) and condition ($p = 0.027$; Figure 6.2b). A larger peak in insulin was observed during EX+PRO compared with CON (EX+PRO: 1374 ± 602 vs CON: 1050 ± 420 pmol·l⁻¹, $p = 0.004$) and insulin AUC was greater during EX+PRO than CON, but not EX, during the acute (0-60 minutes), intermediate (0-120 minutes) and full (0-240 minutes) postprandial analyses (Table 6.3). There were no differences observed between conditions in whole-body insulin sensitivity following breakfast consumption (Matsuda-ISI: EX+PRO: 2.3 ± 0.3 , EX: 2.3 ± 0.3 , CON: 2.6 ± 0.4 ; $p = 0.344$).

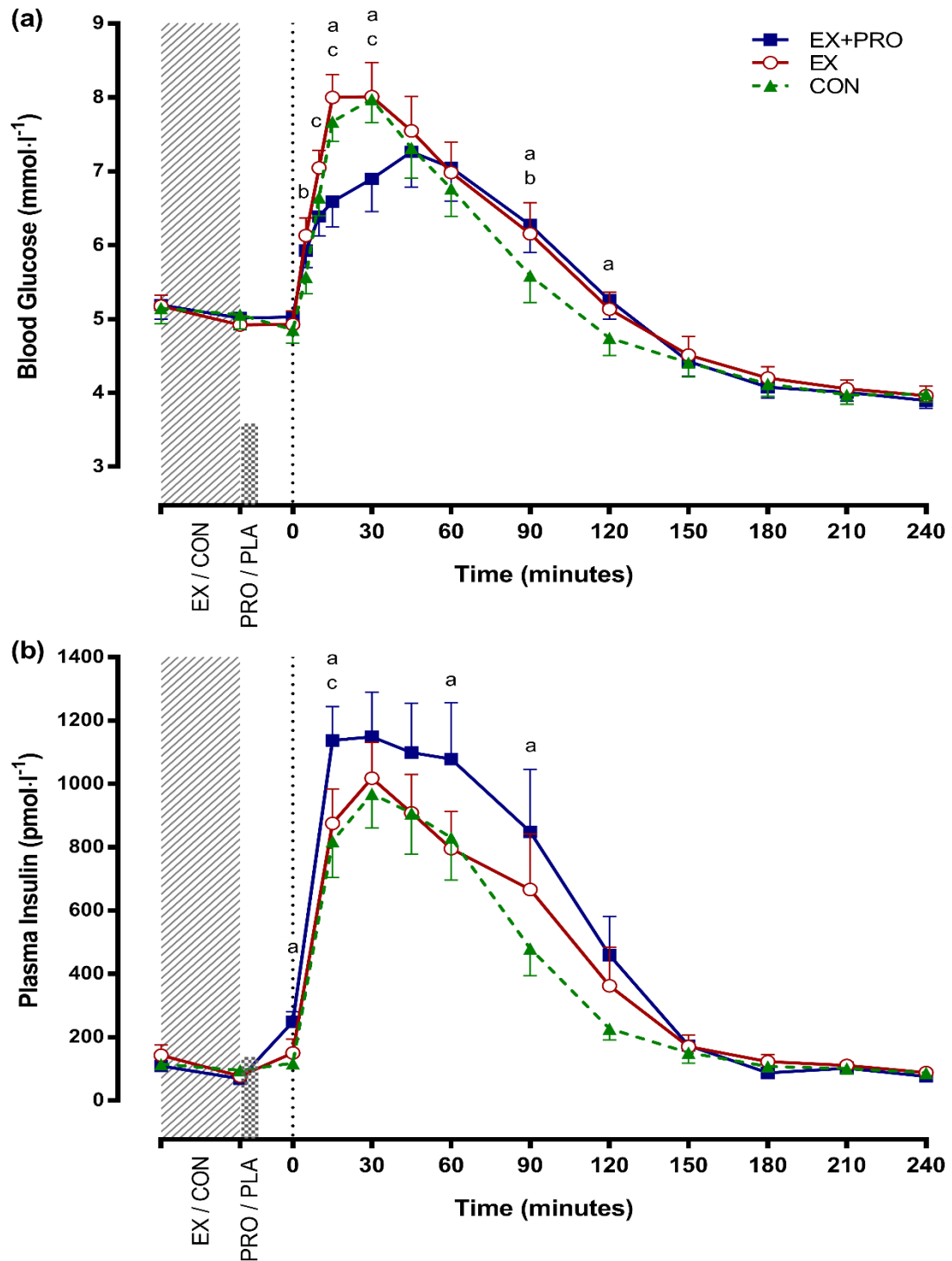


Figure 6.2 Mean \pm SEM ($n = 12$) temporal changes in blood glucose (a) and plasma insulin (b) concentrations. Significant differences ($p < 0.05$) between conditions at individual time points are defined as follows; a, EX+PRO vs CON; b, EX vs CON; c, EX+PRO vs EX. Dotted line indicates time of breakfast consumption. EX+PRO: exercise with whey protein preload trial; EX: exercise trial; CON: resting trial.

Table 6.3 Area under the curve responses during the post-breakfast period for blood and plasma analytes.

	Time (minutes)	Area Under the Curve		
		EX + PRO	EX	CON
Glucose (mmol·min ⁻¹ ·l ⁻¹)	0 – 60	405 ± 22 ^a	446 ± 21 ^b	430 ± 17 ^{a,b}
	0 – 120	778 ± 41	811 ± 39	770 ± 34
	0 – 240	1291 ± 54 ^{a,b}	1330 ± 51 ^a	1276 ± 49 ^b
Insulin (pmol·min ⁻¹ ·l ⁻¹)	0 – 60	60725 ± 7076 ^a	49121 ± 5417 ^{a,b}	47536 ± 5955 ^b
	0 – 120	109248 ± 15805 ^a	86490 ± 11273 ^{a,b}	77807 ± 9885 ^b
	0 – 240	128173 ± 19097 ^a	105374 ± 14341 ^{a,b}	93313 ± 11013 ^b
Triglyceride (mmol·min ⁻¹ ·l ⁻¹)	0 – 60	90.4 ± 9.3	93.9 ± 10.0	89.6 ± 6.1
	0 – 120	187.9 ± 18.8	195.7 ± 21.1	192.4 ± 13.7
	0 – 240	403.6 ± 38.1	412.7 ± 43.4	402.4 ± 29.3
Glycerol (μmol·min ⁻¹ ·l ⁻¹)	0 – 60	3342 ± 288	3226 ± 154	3652 ± 261
	0 – 120	5966 ± 607 ^{a,b}	5656 ± 324 ^a	6912 ± 509 ^b
	0 – 240	13254 ± 1281 ^a	16074 ± 915 ^b	15807 ± 1105 ^b

Data presented as mean ± SEM. Different superscript letters within a row indicates significant difference between conditions ($p < 0.05$). EX+PRO: exercise with whey protein preload trial; EX: exercise trial; CON: resting trial.

6.3.3 Plasma triglyceride and glycerol

There were no differences between conditions at baseline, immediately post exercise, or immediately prior to breakfast in plasma triglyceride concentrations ($p > 0.05$). Following breakfast, responses were significantly affected by time ($p < 0.001$), such that triglyceride was significantly increased above baseline at 120-210 minutes post-breakfast in all conditions (Figure 6.3a; all $p < 0.05$). There was no effect of condition or a condition x time interaction effect on plasma triglyceride responses, and AUC was similar across conditions (Table 6.3; all $p < 0.05$).

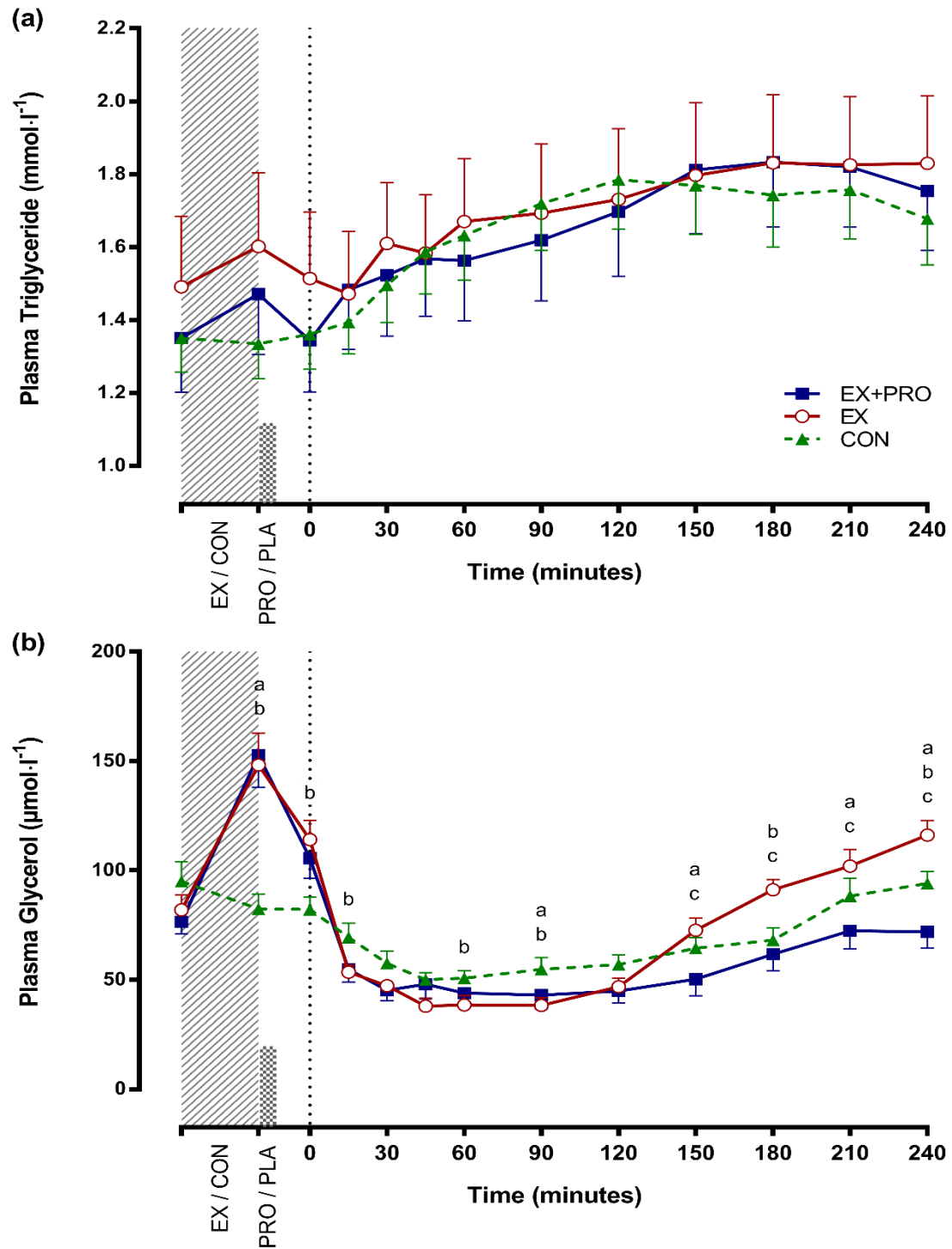


Figure 6.3 Mean \pm SEM ($n = 12$) temporal changes in plasma triglyceride (a) and glycerol (b) concentrations. Significant differences ($p < 0.05$) between conditions at individual time points are defined as follows; a, EX+PRO vs CON; b, EX vs CON; c, EX+PRO vs EX. Dotted line indicates time of breakfast consumption. EX+PRO: exercise with whey protein preload trial; EX: exercise trial; CON: resting trial.

Circulating glycerol concentrations did not differ between conditions at baseline, but were significantly greater in exercise trials than CON immediately post-exercise (Figure 6.3b; both $p \leq 0.004$), remaining elevated immediately prior to breakfast in EX ($p = 0.019$). Post-breakfast glycerol concentrations were influenced by condition ($p = 0.004$), time ($p < 0.001$) and a condition x time interaction effect ($p < 0.001$). Concentrations were similarly suppressed below baseline in EX+PRO and EX at 15-120 minutes post-breakfast, after which EX+PRO remained below EX ($p \leq 0.013$). Suppression of glycerol under CON was more delayed, with levels remaining below baseline at 30-90 minutes post-breakfast ($p \leq 0.006$). AUC over 0-120 minutes was lower in EX than CON ($p = 0.01$), while over the full postprandial period comparison of AUC indicated lower glycerol levels in EX+PRO compared to both EX ($p = 0.023$) and CON ($p = 0.005$; Table 6.3).

6.3.4 Energy balance and substrate oxidation

The rate of energy expenditure did not differ between conditions at baseline ($p = 0.10$). The amount of energy expended did not differ between EX+PRO and EX throughout ($p > 0.05$), but was understandably greater during the exercise period in EX+PRO and EX than CON, fully accounting for the significantly greater total energy expenditure in exercise trials compared with CON (both $p < 0.001$; Table 6.4). No differences were detected between conditions in absolute energy intake at the *ad libitum* lunch meal ($p = 0.886$), signifying that participants did not compensate for the excess energy expended in exercise trials at the subsequent lunch meal. When total intake over the whole trial (breakfast, test drink and lunch) was compared, no between-condition differences were present ($p = 0.491$).

Table 6.4 Energy intake, expenditure and substrate metabolism during the exercise period (30 minutes), post-breakfast period (240 minutes) or full protocol (~300 minutes).

		EX+PRO	EX	CON
Energy Intake (kJ)	Lunch	4623 ± 356	4728 ± 385	4569 ± 343
	Total	6925 ± 356	6686 ± 385	6527 ± 343
Energy Expenditure (kJ)	Exercise period	938 ± 48 ^a	914 ± 43 ^a	203 ± 14 ^b
	Post-Breakfast	1657 ± 87	1695 ± 83	1572 ± 73
	Total	2690 ± 134 ^a	2712 ± 122 ^a	1866 ± 86 ^b
Carbohydrate Oxidation (g)	Exercise period	31.4 ± 2.6 ^a	30.1 ± 2.7 ^a	6.1 ± 1.1 ^b
	Post-Breakfast	55.4 ± 4.3	56.2 ± 5.7	57.1 ± 4.3
	Total	89.3 ± 6.9 ^a	89.0 ± 8.2 ^a	65.5 ± 5.5 ^b
Fat Oxidation (g)	Exercise period	10.7 ± 1.3 ^a	10.7 ± 1.3 ^a	2.9 ± 0.4 ^b
	Post-Breakfast	21.0 ± 2.0	21.7 ± 2.1	18.0 ± 2.1
	Total	33.1 ± 3.3 ^a	33.9 ± 3.5 ^a	22.4 ± 2.6 ^b

Data presented as mean ± SEM. Different superscript letters within a row indicates significant difference between conditions ($p < 0.05$). EX+PRO: exercise with whey protein preload trial; EX: exercise trial; CON: resting trial.

Rates of fat and carbohydrate oxidation did not differ between conditions at baseline ($p = 0.593$ and $p = 0.879$ respectively). Greater amounts of fat and carbohydrate were utilised over the course of each exercise trial in comparison to resting control (all $p < 0.05$), however substrate metabolism was not influenced by consumption of whey protein, with similar fat and carbohydrate oxidation observed between EX+PRO and EX throughout (all $p > 0.05$; Table 6.4). The greater substrate utilisation in exercise trials appears to be exclusively due to the contribution of the exercise bout, as no detectable differences were observed in fat or carbohydrate oxidation between conditions in the postprandial period following breakfast consumption ($p = 0.127$ and $p = 0.969$ respectively).

6.3.5 Subjective appetite ratings

A significant effect of time on combined appetite responses was observed ($p < 0.001$), with appetite decreasing similarly following breakfast in all trials, returning to baseline levels at 90-240 minutes post-breakfast, before decreasing similarly after the *ad libitum* lunch meal (Figure 6.4). There was no detectable difference in AUC for combined appetite score and individual components of subjective appetite under all conditions (all $p > 0.05$; Table 6.5).

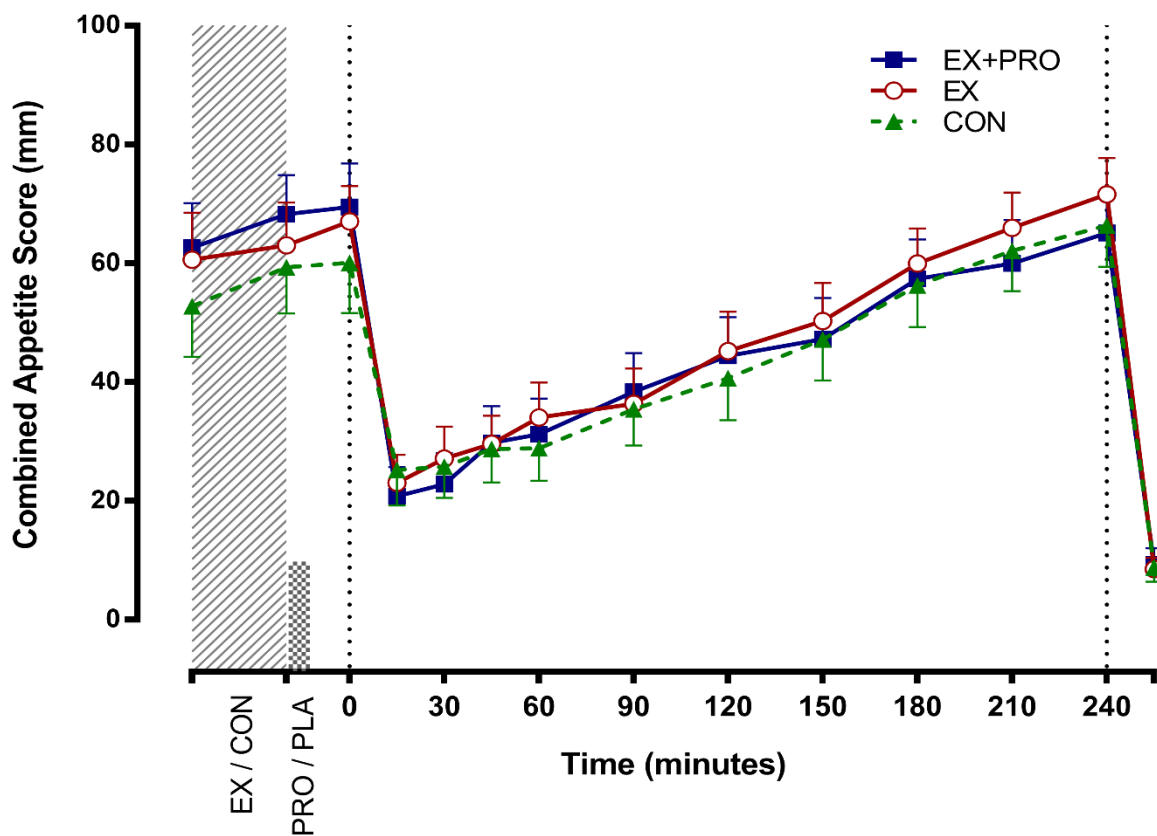


Figure 6.4 Mean \pm SEM ($n = 12$) temporal changes in plasma combine appetite score. Dotted line indicates time of breakfast consumption. EX+PRO: exercise with whey protein preload trial; EX: exercise trial; CON: resting trial.

Table 6.5 Area under the curve responses during the post-breakfast period for subjective appetite responses.

	Area under the curve		
	EX+PRO	EX	CON
Combined appetite (mm·min ⁻¹)	10725 ± 1354	11280 ± 1230	10545 ± 1417
Hunger (mm·min ⁻¹)	9611 ± 1213	10654 ± 1173	9915 ± 1356
Fullness (mm·min ⁻¹)	12931 ± 1399	12886 ± 1318	13870 ± 1423
PFC (mm·min ⁻¹)	11019 ± 1487	11896 ± 1276	11590 ± 1570
Satisfaction (mm·min ⁻¹)	12797 ± 1394	12545 ± 1247	13456 ± 1449

Data presented as mean ± SEM. PFC: prospective food consumption; EX+PRO: exercise with whey protein preload trial; EX: exercise trial; CON: resting trial.

6.4 Discussion

The main finding from this study was that differential effects on postprandial glycaemia were observed when prior low-moderate intensity exercise was followed by supplemental whey protein, compared with the absence of a protein preload. In addition, neither exercise, nor its combination with post-exercise whey protein ingestion, affected postprandial triglyceride responses, subjective perceptions of appetite or the amount of food consumed at a subsequent lunch meal. Glucose tolerance was moderately impaired following prior exercise, indicated by a higher glucose AUC_{0-240 min} when exercise was performed without additional protein compared to resting control, while insulin AUC remained unchanged. Glucose peak and acute AUC were not different between these conditions, indicating that this effect was manifested by a more prolonged elevation in blood glucose after prior exercise. When whey protein was ingested following exercise and prior to breakfast consumption, this impairment

appears to be negated, as glycaemia did not differ from control overall, and was ~9% lower in the acute (0-60 minutes) period following breakfast, with a significant reduction in peak glucose excursion.

The observation of increased glycaemic response immediately following an acute bout of exercise is in accordance with previous findings in healthy trained males (Rose *et al.*, 2001) and obese normoglycaemic males (Knudsen *et al.*, 2014). Interestingly, the latter study reported that post-exercise glucose tolerance was preserved in age and BMI-matched participants with IGT and T2DM, indicating that this effect may be dependent on underlying glycaemic control. Although fasting glucose levels of participants in the current study were in the normal range, HOMA-IR levels indicated an underlying degree of insulin resistance (Geloneze *et al.*, 2006), signifying that the effect observed may have been larger in participants with greater insulin sensitivity. Glucose disposal is increased during exercise in an insulin-independent manner (Goodyear & Kahn, 1998), due to muscle contraction-induced translocation of GLUT4 (Borghouts & Keizer, 2000), an effect that persists for several hours after cessation of exercise (Colberg *et al.*, 2010). Circulating insulin was similar between EX and CON throughout, indicating that increased post-exercise circulating glucose may be explained by greater exogenous and endogenous glucose appearance. Exercise-induced elevation in catecholamine levels may increase hepatic glucose output (Frayn, 2010), and has been shown to enhance the appearance of orally ingested glucose in an animal model via stimulation of sodium-glucose linked transporter type 1 (SGLT1) (Aschenbach *et al.*, 2002). Increased postprandial splanchnic perfusion after exercise may also explain increased glucose absorption (Gonzalez, 2014). Evidence of greater glucose appearance following exercise was reported by Rose *et al.* (2001) who observed a 30% elevation in glucose

appearance during an OGTT following 30 minutes of cycling exercise, albeit at a higher intensity (70% $\dot{V}O_{2peak}$) than the current study.

It is currently unclear how long this effect persists for, however it is likely to be transient in nature (Knudsen *et al.*, 2014), and blood glucose levels were similar between exercise and control trials beyond 120 minutes post-breakfast in the current study. Exercise-induced improvements in insulin sensitivity may subsequently result in improved glucose tolerance for several hours after a single bout of exercise (Bonen *et al.*, 1998), likely due to the drive for muscle glycogen replenishment (Horowitz, 2007). In addition, should obese individuals take part in regular exercise training, then improvements in aerobic fitness and potential weight loss are likely to have independent effects on improving glucose metabolism (Borghouts & Keizer, 2000; Coker *et al.*, 2009). Nevertheless, knowledge of the acute effect of exercise on glucose tolerance when food is ingested immediately or shortly after exercise, is of significance when aiming to ameliorate the adverse effects of postprandial hyperglycaemia on metabolic health (Ceriello *et al.*, 2008; Monnier & Colette, 2015).

The observed reduction in acute glycaemia following post-exercise whey protein supplementation could be attributed to a combination of mechanisms, including the direct effects of amino acids, particularly leucine, on β -cell stimulation (Gao *et al.*, 2003) and activation of the incretin response (Salehi *et al.*, 2012). An increase in plasma insulin in the whey trial compared to control was observed 15 minutes after consumption of the whey preload, signifying greater circulating insulin coinciding with the timing of breakfast consumption. The post-breakfast rise in insulin secretion also occurred earlier in the EX+PRO trial, with higher concentrations observed at 15 minutes post-breakfast compared

with both non-protein conditions. The fact that the acute glucose response was attenuated in EX+PRO compared with EX, in the context of a potentially increased rate of post-exercise glucose absorption, is suggestive of delayed gastrointestinal transport of orally ingested glucose. As described in Chapter 5, the rate of gastric emptying exerts considerable influence in determining the magnitude of postprandial glucose excursions (Marathe *et al.*, 2013; Mignone *et al.*, 2015), accounting for ~35% of variance in glycaemic response to carbohydrate-containing meals in healthy individuals (Horowitz *et al.*, 1993). Large fluctuations in circulating glucose concentrations have deleterious effects on endothelial function and oxidative stress in both healthy individuals and T2DM patients (Ceriello *et al.*, 2008). The attenuation of peak glucose excursion compared to both exercise and resting control conditions in the current study may therefore indicate a role for pre-meal supplementation of whey protein both at rest, and when consuming meals shortly after bouts of low-moderate intensity exercise, in obese individuals. The stimulus was not great enough to detect a reduction in glycaemia over the full postprandial period, however it was sufficient to negate the increase observed in post-exercise glycaemia without additional whey protein.

There was no effect of whey protein supplementation on postprandial triglyceride in the current study. This reflects the findings of Chapter 5 where the same breakfast meal was administered, albeit without prior exercise, and is most likely due to the low fat content of the breakfast meal provided, which contrasts with previous investigations of whey protein and postprandial lipaemia (Holmer-Jensen *et al.*, 2013; Mortensen *et al.*, 2009; Pal *et al.*, 2010b). Elevated glycerol concentrations immediately post-exercise are indicative of increased lipolysis and lipid substrate availability for exercise, confirmed by the observed increase in fat oxidation during exercise. Increased fat oxidation is associated with the

postprandial triglyceride-lowering effects of exercise (Trombold *et al.*, 2014), however postprandial substrate utilisation was not significantly different between exercise and control trials in the present study, and triglyceride responses were similar between conditions. A large body of evidence implicates exercise in the attenuation of postprandial lipaemia (Freese *et al.*, 2014; Maraki & Sidossis, 2013), however the clear majority of studies have administered a test meal >4 hours after cessation of exercise, with those studies that have shown a more acute effect administering meals considerably greater in fat content (>95 g) and prescribing a greater workload during aerobic exercise bouts than the current study (Katsanos & Moffatt, 2004; Plaisance *et al.*, 2008).

The magnitude of postprandial triglyceride reduction appears to be related to the amount of energy expended during the most recent exercise bout (Gill *et al.*, 2002). Participants expended ~926 kJ during 30 minutes brisk walking in the current study, which may be considered a relatively modest energy deficit. This was necessitated by the fact that participants were habitually inactive, obese individuals and the bout was designed to reflect a realistically achievable isolated exercise session within this population. Furthermore, replacement of the exercise-induced energy deficit via mixed-macronutrient (Burton *et al.*, 2008) or carbohydrate (Harrison *et al.*, 2009) feeding attenuates or abolishes improvements in postprandial triglyceridaemia. In the current study, participants consumed breakfast containing more than double the amount of energy expended during treadmill walking, which may account for the similar lipaemic responses in exercise and resting conditions. It has also been observed that consumption of a meal immediately after exercise diminishes the shift from carbohydrate to fat oxidation that usually follows exercise (Dionne *et al.*, 1999), which

may explain the lack of significant differences in substrate utilisation in resting and exercise conditions in this study.

Appetite was unaffected by prior exercise in the current study, which appears to be consistent with previous evidence suggesting that appetite is not altered by acute moderate-intensity exercise (Blundell & King, 2000). In accordance with the comparable subjective appetite responses, lunch meal energy intake 4 hours post-breakfast was similar between conditions. The failure of participants to compensate for the deficit created by prior exercise reflects the findings of the majority of exercise studies (Martins *et al.*, 2008) and brisk walking protocols (King *et al.*, 2010). The moderate energy deficit created by the exercise bout in the present study, in addition to the short duration between exercise and consumption of a standardised breakfast, is likely to have influenced this response. It may also be a possibility that energy expenditure from exercise is gradually compensated for over several meals or even days, however such compensation is likely to only partially account for energy expended (Blundell *et al.*, 2003). Nevertheless, the provision of standard breakfast meals, in addition to the lack of a difference in lunch intake, meant that participants maintained a difference in energy balance in the exercise control trial compared with resting control that was equivalent to the net amount expended during exercise. This highlights the potential efficacy of brisk walking to create acute energy deficits in obese individuals and, if repeated regularly, a potential application for weight control.

A limitation of the current study is that a whey protein supplementation trial without prior exercise was not conducted, which may have allowed uncoupling of the interaction of these two factors on postprandial responses. However, having identified the effectiveness of a

whey protein preload to attenuate acute postprandial glycaemia in the previous chapter, the present study was designed to investigate the potential of whey protein to influence post-exercise effects, rather than the effects of whey protein *per se*. In addition, implementation of a longer investigation period may have been warranted, as the transient effects of a single bout of exercise on insulin sensitivity may last for up to 72 hours (Colberg *et al.*, 2010), indicating that these beneficial effects may occur beyond the time frame studied here. The timing of post-exercise feeding may have limited the ability to identify significant effects of exercise on postprandial lipaemia and subsequent intake, however the consumption of a meal or snack following fasted morning exercise is likely, ensuring that these findings hold relevance in a free-living setting.

In summary, this study in sedentary, overweight males showed that an isolated bout of brisk walking exercise moderately impaired post-exercise glucose tolerance, and that a whey protein preload consumed immediately post-exercise negates this effect. Furthermore, acute postprandial glycaemia was attenuated following whey protein consumption, and energy intake at a later lunch meal was not influenced by the intervention. Caution should be applied when interpreting the acute effects of a single bout of exercise given the benefits associated with regular exercise training.

CHAPTER 7

General Discussion

7.1 Introduction

Chronic metabolic conditions such as T2DM currently place an unprecedented burden upon global health care systems (Diabetes UK, 2014; Wang *et al.*, 2011), and vast swathes of scientific literature are devoted to understanding their aetiology and the development of strategies for prevention and treatment. In this context, the postprandial period is of significant interest to researchers due to the acute effects of meal handling on the metabolic and hormonal milieu, and the adverse health effects associated with chronic exposure to an impaired response. Given that western populations effectively spend most the day in the postprandial state, interventions aiming to adjust the acute meal response are pertinent to preventing the deterioration in metabolic health that may precede the onset of overt symptoms. Several studies have indicated potential health-promoting effects of whey protein supplementation, however a large degree of variation exists in the methodology used. The research presented in this thesis has built on existing knowledge by examining the impact of whey protein ingestion at breakfast on acute and subsequent meal postprandial metabolism and appetite, utilising practical supplementation strategies that provide the research with ‘real-world’ application. The purpose of this chapter is to collate and reflect upon the main findings, consider the limitations of the methodology utilised and provide directions for future research.

7.2 Reflections on main findings

7.2.1 Whey protein supplementation and glycaemia

Based on previous literature, the potential for whey protein to positively influence postprandial blood glucose is clear. A variety of designs have reported reduced glycaemia following consumption of whey protein as a standalone supplement or as part of a test meal in normal-weight (Akhavan *et al.*, 2010; Gunnerud *et al.*, 2013), overweight (Bowen *et al.*, 2006b; Pal *et al.*, 2010b) or T2DM populations (Jakubowicz *et al.*, 2014; Ma *et al.*, 2009)

From this series of studies it would appear that 20 g whey protein is sufficient to reduce postprandial glycaemia in obese but not normal-weight males, however this may not be a fair comparison to make due to the different designs conducted in these populations. In Chapter 5 the supplementation of whey as a preload 15 minutes prior to breakfast was the most effective intervention emphasised by reduced post-breakfast glucose AUC, while insulin remained at similar levels to control. This strategy was not tested in the normal-weight participants in Chapter 4, where whey was consumed at the same time as the breakfast meal. The use of a different breakfast meal also makes direct comparison difficult, however the GI of breakfast meals used in Chapters 4a and 5 were matched.

The importance of the timing of supplementation is illustrated by the post-breakfast glycaemic responses in all three protein conditions in Chapter 5 (Figure 5.2b). Although not all significantly different from each other, glycaemia appears to increase in a stepwise fashion as whey supplementation progresses from -15 to 0 and +15 minutes in relation to the timing

of breakfast consumption, indeed a straight line could almost be drawn through the graphical representation to illustrate this. When comparing the with-meal whey protein trial from Chapter 5 with the equivalent condition in Chapter 4a, findings between normal-weight and obese participants do not differ markedly. Post-breakfast AUC did not differ from the non-protein control in either comparison, and peak glucose excursion was significantly lower in obese participants while in normal-weight participants there was a non-significant trend for reduced peak glucose ($p = 0.059$). This makes it attractive to speculate that if the normal-weight participants had completed the same protocol as that implemented in Chapter 5, significant reductions in glucose AUC and peak glucose may also have been observed. Only one acute study has focussed on the glycaemic response to whey protein in both normal-weight and overweight participants (Zafar *et al.*, 2013). Whey protein was effective in reducing postprandial glycaemia in both groups, although glucose was attenuated from a greater peak in overweight participants, with speculation that insulin sensitivity is enhanced following whey supplementation to a greater extent in overweight individuals, as observed following chronic whey protein consumption in both rats (Belobrajdic *et al.*, 2004) and humans (Pal *et al.*, 2010a).

It may be argued that reducing postprandial glycaemia is of less importance in the normal-weight cohort due to the healthy weight status and relative lack of insulin resistance exhibited by these participants (HOMA-IR: 2.1 ± 1.1). However, postprandial hyperglycaemia contributes to CVD risk independently of weight status, (Bianchi *et al.*, 2008; Gerich, 2006; Monnier *et al.*, 2012), even when fasting glucose is in the normal range (Ning *et al.*, 2012). Ensuring that postprandial glycaemic excursions remain within a healthy range throughout

the life course may therefore be a significant strategy in averting the onset of metabolic disease.

The finding that post-exercise whey protein negated the impairment observed in post-exercise glycaemia in physically inactive obese males was a novel and interesting outcome. However, the lack of a non-exercise whey protein condition in this study made uncoupling of the effects of whey protein and prior exercise on glycaemia problematic. Whether the blood glucose response would be further reduced by consuming a whey protein preload in the absence of prior exercise cannot be answered with any degree of certainty, however overlaying the trace of this condition from Chapter 5 onto the glucose trace from Chapter 6 (Figure 7.1) appears to confirm this conjecture. Only five participants took part in both studies, making this a speculative observation, however the breakfast meal, whey protein dose and blood sampling schedule were identical across studies, albeit participants were observed for an additional hour in Chapter 6. Examination of these responses confirms the moderate impairment in glucose tolerance following an acute bout of brisk walking exercise, and the subsequent abrogation of this effect by post-exercise whey protein consumption. Although this protocol was not completed by the normal-weight participants in Chapter 4, this effect may hold relevance, despite the greater level of fitness, as previous studies have reported higher post-exercise glycaemia in healthy trained males (Rose *et al.*, 2001) and obese normoglycaemic males (Knudsen *et al.*, 2014). Although this is likely to be a transient effect, this phenomenon may be of significance to those who undertake exercise infrequently, and when intake of a meal is likely in the immediate post-exercise period.

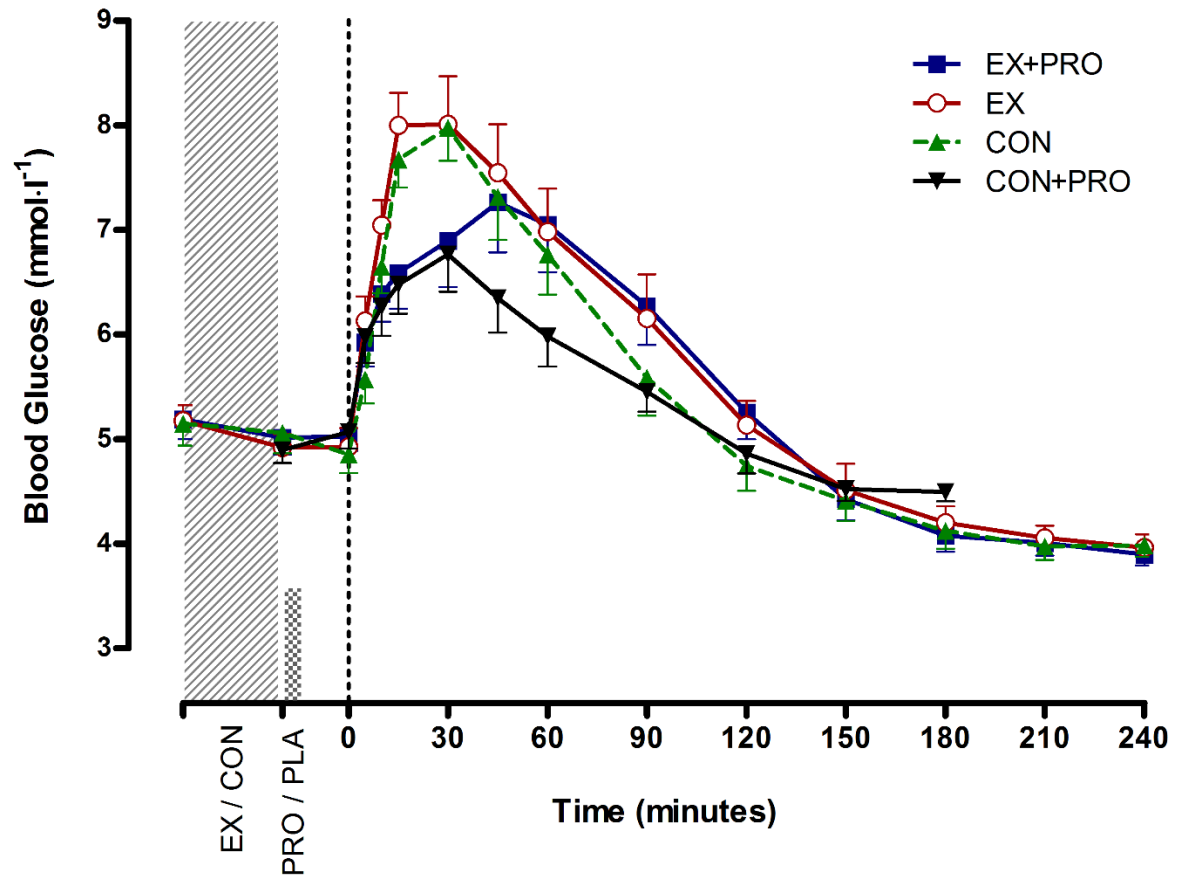


Figure 7.1 Mean \pm SEM temporal changes in blood glucose concentrations. Dotted line indicates time of breakfast consumption. EX+PRO: exercise with whey protein preload trial; EX: exercise trial; CON: resting trial; CON+PRO: resting trial with whey protein preload.

When assimilating the findings reported in this thesis in the context of postprandial glycaemia, the rationale for further investigation of the glucose reducing effects of whey protein is prominent, particularly in centrally-obese individuals. Although a whey preload has been observed to be particularly effective in reducing acute postprandial glycaemia, further investigation of the optimal timing of pre-meal ingestion may be warranted. To the author's knowledge this was the first study to assess the effect of timing of whey protein consumption on post-meal glycaemia in a non-diabetic population. The modification of post-

exercise glycaemia has also been observed following brisk walking in previously sedentary obese individuals, however it remains to be seen whether such a response would be observed when varying the modality or intensity of exercise, or in different populations. Furthermore, Chapters 4 and 5 appeared to confirm that supplementation of 20 g whey protein with a meal does not affect glycaemia at the next meal or later in the day, potentially providing a rationale for the investigation of supplementation with multiple sequential meals.

7.2.2 Whey protein supplementation and lipaemia

The importance of postprandial hypertriglyceridaemia as a determinant of CVD risk is increasingly recognised (Kolovou *et al.*, 2011), and the beneficial effects of whey protein supplementation on post-meal triglyceride reported in some studies (Holmer-Jensen *et al.*, 2013; Mortensen *et al.*, 2009; Pal *et al.*, 2010b) is of interest for this reason. Nevertheless, evidence is currently limited to comparison of whey with glucose or other protein sources, and studies investigating this response in the absence of unrealistically high-fat meals have been lacking.

The consumption of whey protein with a high-carbohydrate (Chapter 4a) or high-fat (Chapter 4b) breakfast meal in normal-weight participants, or a mixed breakfast meal (Chapters 5 and 6) in centrally-obese participants, did not modify postprandial triglyceride responses throughout this body of work. It could be speculated that the low-fat content of the breakfasts served in Chapter 4a (2 g) and Chapters 5 and 6 (9 g) would not be expected to augment a large enough postprandial response for any reduction to be apparent, and this was almost

certainly the case in these studies. When supplemented with a fat-rich meal (Chapter 4b), whey protein actually appeared to induce a moderately higher triglyceride response, however the difference in total AUC (~15%) was not significant. This may be explained by the reduced post-lunch insulin sensitivity observed in the whey protein trial, as insulin resistance is associated with impaired clearance and increased production of chylomicrons (Pal *et al.*, 2010b) thus increasing triglyceride exposure. Considering the higher fasting triglyceride and HOMA-IR levels of the centrally-obese cohorts from Chapters 5 and 6, differential responses may be exhibited compared with their normal-weight counterparts, and future research should investigate this response. Evidence from these studies adds to the mixed picture regarding the potential of whey protein to improve markers of dyslipidaemia. In addition, prior low-moderate intensity exercise did not appear to affect postprandial triglyceride levels, although delaying the consumption of the post-exercise meal may allow identification of this response in future work.

7.2.3 Whey protein supplementation and appetite

Although not measured in this body of work, whey protein has previously been reported to augment postprandial levels of gut hormones including GLP-1 and CCK in addition to suppressing ghrelin (Bowen *et al.*, 2006a), thus promoting satiety. The effect on subjective appetite in previous studies is equivocal, however whey protein has been shown to suppress subsequent food intake with greater consistency. In this thesis, whey protein did not influence subjective appetite regardless of the population studied or the interventions tested. In addition, subsequent energy intake at lunch was not affected by prior exercise or post-exercise whey protein. These findings are perhaps surprising as whey has consistently been

shown to potentiate insulin secretion throughout the studies in this thesis, and the short-term satiating effect of insulin has previously been reported (Flint *et al.*, 2007), however this effect may be blunted in obese individuals. Whether whey protein would influence satiety outside of the laboratory remains uncertain, and future work should investigate this response further in the context of preventing overconsumption and subsequent obesity. The exercise-induced energy deficit generated by participants in Chapter 6 was not compensated for at a subsequent lunch meal, independent of whey supplementation, which was consistent with previous research (Blundell & King, 2000). Brisk walking and post-exercise whey protein consumption may therefore be an effective combination in reducing chronic disease risk, enabling the creation of moderate energy deficits while controlling postprandial glycaemic excursions.

7.3 Methodological limitations

Several limitations have been addressed within the relevant experimental chapters of this thesis, however there are a number of common limitations which may apply across all chapters. In order to assess postprandial responses, studies were confined to the controlled environment of the laboratory. Whilst this allows thorough investigation of effects and mechanisms of interest without the confounding influence of many extraneous factors, it limits the application of findings in the free-living setting. Although interstitial glucose was recorded outside of the laboratory in Chapter 4, this did not constitute free-living conditions as participants were asked to replicate dietary intake across conditions. Efforts were made to increase the ecological validity of findings throughout, including administering doses of protein that could realistically be supplemented alongside a meal, and using foods that were

typical of those consumed at breakfast and lunch meals across the population. However, due to the influence of behavioural and environmental factors on satiety (Benelam, 2009), measures such as subjective appetite and *ad libitum* energy intake may still be affected by the unfamiliar environment. Care should therefore be taken when interpreting the relevance of these findings for individuals in the free-living setting.

Extrapolation of findings may also be hindered by the relatively homogenous nature of the sample groups within each study. Typical of a number of studies conducted in this area of research, only male participants were used. This was a consequence of the desire to eliminate the confounding hormonal influence of the menstrual cycle upon metabolism (Brennan *et al.*, 2009) which, if controlled for, may have added considerably to the duration of each data collection period during these multiple-visit designs. In addition, although no screening for ethnicity was in place throughout this work, all participants were of European ethnicity except for two who were of South Asian ethnicity. It is well established that differences in disease risk exist between ethnicities, with higher prevalence of T2DM amongst South Asian populations (Khunti *et al.*, 2013) a well-reported example. Thus, extrapolation of findings to other populations should be interpreted cautiously.

The findings of this body of work may have been enhanced by the measurement of additional parameters to fully explain the mechanisms underlying the effects observed. Measurement of energy substrates and insulin enabled patterns of metabolic regulation to be detected, however this evidence was insufficient to fully explain the influence of whey protein on glycaemia observed in Chapters 5 and 6. Determination of circulating concentrations of amino acids, incretin hormones and DPP-IV, as well as rates of gastric emptying, may have

been beneficial in this respect. Implementation of such measures was limited by practical constraints in the studies described, however consideration should be given to incorporating these measures into future work.

7.4 Future directions

Acute trials such as those reported in this thesis, provide valuable information regarding the effects of whey protein consumption on immediate post-meal responses in a laboratory environment. As identified in Chapters 4 and 5, whey protein does not appear to significantly influence postprandial metabolism at a subsequent eating occasion. In order to identify the efficacy of whey to influence glycaemia and other markers over a longer period, studies should be conducted to investigate the effects of supplementation at multiple meals. Frid *et al.* (2005) reported the effects of supplementing breakfast and lunch meals with whey protein in individuals with T2DM however, to the author's knowledge, no studies have been conducted in other populations or investigated supplementation at more than two consecutive meals.

In addition, consideration should be given to the fact that prevention of deteriorating metabolic health may require chronic improvements in postprandial glycaemia and other markers which cannot be observed in the acute laboratory setting. Despite this, studies assessing the longer-term effects of whey supplementation are relatively few in number, with only one study having been carried out in normal-weight participants (Astbury *et al.*, 2014) and a small number in overweight/obese (Baer *et al.*, 2011; Gouni-Berthold *et al.*, 2012; Pal *et al.*, 2010a; Pal *et al.*, 2014) or diabetic (Ma *et al.*, 2015) individuals. Although some

inconsistencies are apparent, the limited evidence to date appears to show that chronic supplementation of the diet with whey protein is associated with metabolic health benefits including improved fasting lipid profile and insulin sensitivity, with possible effects on food intake and body mass. This work may be advanced through development of an optimal chronic supplementation strategy, while development of food products incorporating whey protein may enhance adherence to supplementation. The use of CGM technology would also enable assessment of the longer-term influence of whey protein supplementation on mean glycaemic exposure and glycaemic variability.

Additionally, the implementation of longer-term supplementation protocols would allow assessment of potential negative consequences of whey protein supplementation on metabolic health. Increased postprandial insulinaemia in the absence of a reduction in glycaemia is suggestive of compromised insulin sensitivity, an effect that has previously been observed following acute whey protein ingestion (Smith *et al.*, 2015). Thus there is potential for the chronic exposure to the insulinotropic effects of whey protein to have a desensitising effect on sites of insulin action, which would have a detrimental effect on the prevention of metabolic disease.

7.5 Conclusion

In summary, the findings of this thesis indicate that addition of a practical dose of whey protein to a typical breakfast meal improves the glycaemic response to that meal in centrally-obese males. This may add weight to previously observed inverse associations between consumption of dairy products and obesity, metabolic syndrome and T2DM. Pre-meal whey

protein may modify postprandial glycaemia to a greater extent than consumption during or after a meal, however beneficial effects do not persist past a subsequent meal. In addition, post-exercise whey protein is effective in abolishing the transient impairment in postprandial glycaemia observed following prior walking exercise in previously sedentary individuals. Further investigation is now required to determine the chronic and long-term effects of whey protein consumption and its role in the management of body mass and metabolic regulation.

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APPENDICES

APPENDIX A: Participant information form



PARTICIPANT INFORMATION

The purpose of this information sheet is to provide you with sufficient information so that you can then give your informed consent. It is thus very important that you read this document carefully, and raise any issues that you do not understand with the investigator.

Project Title: Metabolic and appetite responses to a whey protein preload following prior exercise in overweight and obese males

Principal Investigator: Dean Allerton

Supervisor: Dr. Penny Rumbold

Email address: d.allerton@northumbria.ac.uk

INFORMATION FOR POTENTIAL PARTICIPANTS

1. What is the purpose of the project?

Consuming whey protein may have beneficial effects on health, principally by having an impact on blood sugar metabolism, but also by affecting appetite. The purpose of this project is to investigate the effect of consuming whey protein following a bout of low/moderate intensity exercise (brisk walking) on glucose and fats in the blood as well as on appetite.

2. Why have I been selected to take part and what are the exclusion criteria?

You have been asked to participate because you are male, aged between 18 and 55 years old, have a BMI above 25 kg/m², have a waist circumference greater than 102 cm and currently have a low level of physical activity.

You should **NOT** take part if you are a smoker or have any metabolic disorder (i.e. type 1 diabetes, type 2 diabetes, thyroid disorders). If you have a food allergy/intolerance, eating disorder, current illness, regularly skip breakfast or are currently taking medication which may affect your metabolism you should also not take part.

3. Do I have to take part?

Participation is completely voluntary. Before you decide to take part we will discuss the study and you will have the opportunity to ask any questions. If you agree to take part, we will then ask you to sign a consent form. However, if at any time you decide you no longer wish to take part in the study you are free to withdraw at any time without giving a reason.

4. What will I have to do?

After giving informed consent, you will be screened for eligibility to take part in the study. This will involve basic measures being taken (height/weight/waist) and completion of a screening questionnaire and physical activity questionnaire. If eligible, you will be required to visit the lab for 1 short preliminary visit (30 minutes) and 3 separate main trials, each separated by at least 5 days. Each trial will last approximately 6 hours, however you will have the opportunity to read or use a laptop during this time.

Preliminary visit (30 minutes)

The preliminary visit involves walking on a treadmill for 12 minutes, starting with a 3 minute warmup and then completing 3 stages (3 minutes each). At the end of each stage the speed will be increased slightly so that you will be walking at a brisk pace by the end of the test. Throughout the test, you will be required to wear a mask in order to collect the air that you breathe out and a chest strap to record heart rate. You will be provided with a ready meal to eat on the night before each trial.

Main trials (3 x 6 hours)

You will be asked to arrive at the laboratory at the same time of morning for all four trials, having not had breakfast, and having refrained from alcohol and caffeine on the previous day. A cannula will be inserted into a vein in the arm by a trained researcher and a blood sample will be taken. A very small fingertip sample will also be taken. We will also record the air you breathe for a 10 minute period while you rest, and will ask you to collect a sample of urine when you need to use the bathroom.

After this you will be asked to remain seated for 30 minutes (in 1 of the trials) or walk for 30 minutes on a treadmill (in 2 of the trials). The speed will be set at a low/moderate intensity for the walking exercise. 15 minutes after this you will be given a drink which will contain either flavoured water or whey protein, followed 15 minutes later by breakfast which will consist of porridge sweetened with honey. For the following 4 hours you will be required to rest, and at various points we shall collect blood from the cannula that is already placed in the arm and from a fingertip. We shall also ask you to answer questions about your appetite, and will ask you to put on the mask that collects your expired air for a short period every half an hour.

After this you will be provided with lunch which shall consist of pasta in a cheese and tomato sauce, as well as water to drink. You will be asked to keep eating until you feel that you are comfortably full. The cannula will then be removed and you will be able to leave the lab. During the whole visit you will be able to read or use a laptop (Wi-Fi is provided) to pass the time.

5. Will my participation involve any physical discomfort?

The collection of blood via cannulation may result in a small amount of light bruising, however this is normal and will be minimised as this procedure will be carried out by trained personnel. Capillary blood sampling may cause very slight discomfort when the finger-prick technique is used, however this is extremely short-term in nature and usually relatively painless.

6. Will my participation involve any psychological discomfort or embarrassment?

No

7. Will I have to provide any bodily samples (i.e. blood, saliva)?

We will require venous blood samples taken from a cannula at various intervals throughout the day. The maximum amount of blood we will take during the whole day is 130ml, which falls well within safe guidelines for blood sampling, however this may affect your ability to donate blood in the weeks following the study. Capillary blood samples shall also be collected using the standard finger-prick technique. This involves collecting a small droplet of blood from the fingertip. Urine samples shall also be collected during each visit.

8. How will confidentiality be assured and who will have access to the information that I provide?

Upon participation you will be allocated a participant code that will be used to identify your data, therefore your name will not be associated with any data collected. All data will either be kept in a locked filing cabinet or accessed via a password-protected computer. This is in accordance with the Data Protection Act.

Only the principal investigator and their supervisory team will have access to information that you provide, and all records will remain confidential. Any published results will be anonymous, therefore your personal data will not be identifiable.

9. How will my information be stored / used in the future?

All identifiable data will be kept in a locked filing cabinet. In accordance with the Data Protection Act, data will not be kept longer than is necessary for the purposes of this study. Following this it will be destroyed appropriately. Blood samples collected and stored during this study will be disposed of following the conclusion of the study.

10. What will happen to the results of the project?

Data may be included as part of a thesis submission as well as used for publication in the form of a scientific paper or presented at a conference.

11. Who has reviewed the project?

This study has been approved by the Faculty of Health and Life Sciences Research Ethics Committee, Northumbria University.

12. Will I receive any financial rewards / travel expenses for taking part?

Yes. You will receive £150 in compensation for your time after participating fully in this research project. You will also be provided with breakfast and lunch during each trial of the study as well as evening meals the night before each trial.

13. How can I withdraw from the project?

You can withdraw from the project at any point without providing reasons for doing so, by contacting the principal investigator (contact details at the top of this form). If you have any concerns please feel free to discuss them with the principal investigator. If, for any reason, you wish to withdraw your data please contact a member of the research team who will facilitate this, and discuss with you how you want your data to be treated. Please bear in mind that if this may not always be possible if the request is made after a long period following participation, as results may already have been published, however you will not be identifiable in any way from the published data.

14. What happens if there is a problem?

If you have a concern about any aspect of the study you should ask to speak to the researchers who will do their best to answer any questions you may have. If they are unable to resolve your concern, or you wish to make a complaint regarding the study, please contact the chair of the Faculty of Health and Life Sciences Ethics Committee:

Chair of Faculty of Health and Life Sciences Ethics Committee,
Northumberland Building,
Northumbria University,
Newcastle upon Tyne,
NE1 8ST

15. Who is funding and organising the project?

The study is funded by the Faculty of Health and Life Sciences, and organised by researchers within the Department of Sport, Exercise and Rehabilitation at Northumbria University.

16. If I require further information who should I contact and how?

If you would like to ask questions or gain further information on the study please contact the principal investigator on the contact email address listed at the beginning of this form. If you would like to withdraw your data or register a complaint please contact the chair of the ethics committee on the address listed in section 14 of this form.

APPENDIX B: Informed consent form



INFORMED CONSENT FORM

Project Title: Metabolic and appetite responses to a whey protein preload following prior exercise in overweight and obese males

Principal Investigator: Dean Allerton

Participant Code: _____

*please tick
where applicable*

I have read and understood the Participant Information Sheet. ☐

I have had an opportunity to ask questions and discuss this study and I have received satisfactory answers. ☐

I understand I am free to withdraw from the study at any time, without having to give a reason for withdrawing, and without prejudice. ☐

I agree to take part in this study. ☐

I would like to receive feedback on the overall results of the study at the email address given below. ☐

Email address.....

Signature of participant..... Date.....
(NAME IN BLOCK LETTERS).....

Signature of researcher..... Date.....
(NAME IN BLOCK LETTERS).....

APPENDIX C: Health screening questionnaire



Participant Screening

Strictly Confidential

Please answer these questions truthfully and completely. The sole purpose of this questionnaire is to ensure that you are fit and healthy to follow the proposed research programme.

For the participant to complete:

Screening Number	
Date of Birth	
Age	
Gender	Male / Female

How often do you eat breakfast?	Always / Regularly / Sometimes / Rarely / Never
--	---

Do you have any known allergies or intolerances?	Yes / No
If yes please list:	

Do you have or suffer from:		Details:
History of heart disease (e.g. Heart attack, surgery, angina, etc.)	Yes / No	
Problems with the circulation	Yes / No	
High blood pressure	Yes / No	
Diabetes	Yes / No	
Lung disease/breathing problems (e.g. Asthma, COPD, etc.)	Yes / No	
Kidney disease	Yes / No	
Have you ever suffered from:		Details:
Discomfort in the chest, jaw, neck, back or arms	Yes / No	
Light headedness, dizziness or fainting	Yes / No	
Shortness of breath at rest	Yes / No	

Have you had a recent illness?	Yes / No
If yes please list:	

Are you currently taking any medication?	Yes / No
If yes please list:	

Do you have any injuries / joint problems which would make exercising difficult?	Yes / No
If yes please list:	

Are you currently a smoker?	Yes / No	
Have you previously been a smoker?	Yes / No	
If yes:	How long is it since you stopped?	

Is there anything that you feel would stop you from successfully completing the research study outlined to you? <i>If yes please feel free to discuss with the research team</i>	Yes / No
---	----------

For the researcher to complete:

Height (cm)			
Body Mass (kg)			
BMI (kg/m ²)		BMI Classification	
Waist Circumference (cm)			
Hip Circumference (cm)			

IPAQ physical activity level	Low / Moderate / High
Adequate venous access	Yes / No

The information I have given is correct to the best of my knowledge at the time of completion.

Participant Signature	
Date	
Researcher Signature	
Date	

APPENDIX D: International physical activity questionnaire (IPAQ)

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the **last 7 days**. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

Think about all the **vigorous** and **moderate** activities that you did in the **last 7 days**. **Vigorous** physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. **Moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal.

PART 1: JOB-RELATED PHYSICAL ACTIVITY

The first section is about your work. This includes paid jobs, farming, volunteer work, course work, and any other unpaid work that you did outside your home. Do not include unpaid work you might do around your home, like housework, yard work, general maintenance, and caring for your family. These are asked in Part 3.

1. Do you currently have a job or do any unpaid work outside your home?

☐ Yes

☐ No



Skip to PART 2: TRANSPORTATION

The next questions are about all the physical activity you did in the last 7 days as part of your paid or unpaid work. This does not include traveling to and from work.

2. During the last 7 days, on how many days did you do **vigorous** physical activities like heavy lifting, digging, heavy construction, or climbing up stairs as part of your work? Think about only those physical activities that you did for at least 10 minutes at a time.

_____ days per week

☐

No vigorous job-related physical activity



Skip to question 4

3. How much time did you usually spend on one of those days doing **vigorous** physical activities as part of your work?

_____ hours per day
_____ minutes per day

4. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do **moderate** physical activities like carrying light loads as part of your work? Please do not include walking.

_____ days per week

☐

No moderate job-related physical activity



Skip to question 6

5. How much time did you usually spend on one of those days doing moderate physical activities as part of your work?

_____ hours per day
_____ minutes per day

6. During the last 7 days, on how many days did you walk for at least 10 minutes at a time as part of your work? Please do not count any walking you did to travel to or from work.

_____ days per week

☐

No job-related walking



Skip to PART 2: TRANSPORTATION

7. How much time did you usually spend on one of those days walking as part of your work?

_____ hours per day
_____ minutes per day

PART 2: TRANSPORTATION PHYSICAL ACTIVITY

These questions are about how you traveled from place to place, including to places like work, stores, movies, and so on.

8. During the last 7 days, on how many days did you travel in a motor vehicle like a train, bus, car, or tram?

_____ days per week

☐

No traveling in a motor vehicle



Skip to question 10

9. How much time did you usually spend on one of those days traveling in a train, bus, car, tram, or other kind of motor vehicle?

_____ hours per day
_____ minutes per day

Now think only about the bicycling and walking you might have done to travel to and from work, to do errands, or to go from place to place.

10. During the last 7 days, on how many days did you bicycle for at least 10 minutes at a time to go from place to place?

_____ days per week

☐

No bicycling from place to place



Skip to question 12

11. How much time did you usually spend on one of those days to bicycle from place to place?
- _____ hours per day
_____ minutes per day
12. During the last 7 days, on how many days did you walk for at least 10 minutes at a time to go from place to place?
- _____ days per week
- ☐ No walking from place to place → *Skip to PART 3: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY*
13. How much time did you usually spend on one of those days walking from place to place?
- _____ hours per day
_____ minutes per day

PART 3: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY

This section is about some of the physical activities you might have done in the last 7 days in and around your home, like housework, gardening, yard work, general maintenance work, and caring for your family.

14. Think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do vigorous physical activities like heavy lifting, chopping wood, shoveling snow, or digging in the garden or yard?
- _____ days per week
- ☐ No vigorous activity in garden or yard → *Skip to question 16*
15. How much time did you usually spend on one of those days doing vigorous physical activities in the garden or yard?
- _____ hours per day
_____ minutes per day
16. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do moderate activities like carrying light loads, sweeping, washing windows, and raking in the garden or yard?
- _____ days per week
- ☐ No moderate activity in garden or yard → *Skip to question 18*

17. How much time did you usually spend on one of those days doing moderate physical activities in the garden or yard?
- _____ hours per day
_____ minutes per day
18. Once again, think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do moderate activities like carrying light loads, washing windows, scrubbing floors and sweeping inside your home?
- _____ days per week
- ☐ No moderate activity inside home → *Skip to PART 4: RECREATION, SPORT AND LEISURE-TIME PHYSICAL ACTIVITY*
19. How much time did you usually spend on one of those days doing moderate physical activities inside your home?
- _____ hours per day
_____ minutes per day

PART 4: RECREATION, SPORT, AND LEISURE-TIME PHYSICAL ACTIVITY

This section is about all the physical activities that you did in the last 7 days solely for recreation, sport, exercise or leisure. Please do not include any activities you have already mentioned.

20. Not counting any walking you have already mentioned, during the last 7 days, on how many days did you walk for at least 10 minutes at a time in your leisure time?
- _____ days per week
- ☐ No walking in leisure time → *Skip to question 22*
21. How much time did you usually spend on one of those days walking in your leisure time?
- _____ hours per day
_____ minutes per day
22. Think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do vigorous physical activities like aerobics, running, fast bicycling, or fast swimming in your leisure time?
- _____ days per week
- ☐ No vigorous activity in leisure time → *Skip to question 24*

23. How much time did you usually spend on one of those days doing vigorous physical activities in your leisure time?
- _____ hours per day
_____ minutes per day
24. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do moderate physical activities like bicycling at a regular pace, swimming at a regular pace, and doubles tennis in your leisure time?
- _____ days per week
- ☐ No moderate activity in leisure time ➡ *Skip to PART 5: TIME SPENT SITTING*
25. How much time did you usually spend on one of those days doing moderate physical activities in your leisure time?
- _____ hours per day
_____ minutes per day

PART 5: TIME SPENT SITTING

The last questions are about the time you spend sitting while at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading or sitting or lying down to watch television. Do not include any time spent sitting in a motor vehicle that you have already told me about.

26. During the last 7 days, how much time did you usually spend sitting on a weekday?
- _____ hours per day
_____ minutes per day
27. During the last 7 days, how much time did you usually spend sitting on a weekend day?
- _____ hours per day
_____ minutes per day

This is the end of the questionnaire, thank you for participating.

APPENDIX E: Food diary

24 Hour Food Diary and Activity Log



Participant ID: _____ Date: _____

Instructions for recording a Food Diary

There are a lot of considerations to be made when recording your food and fluid intakes. The most important rule is that you are entirely truthful. Your assessment and the feedback we provide is based on what you first provide for us. Being dishonest about the foods that you eat or their amounts mean that the information will be incorrect and therefore we cannot offer you the advice you need. Another important aspect to remember when successfully completing a food diary is that the more information about the preparation of the food and method of cooking, its description and the quantity that you eat, the better our interpretation of your overall diet will be. This means that where possible, weighing the food you eat (using scales provided) and noting the brand and information on packaging of products is extremely helpful, and will lead to the most accurate evaluation of your diet. Additionally make sure to only note what you have eaten. For example if you cannot finish a meal, either record the weight that you have eaten, or state what you have left. Finally, if using a recipe it is useful to provide a copy along with your diary.

Instructions for recording Physical Activity

Please fill in any information regarding exercise you do in this log. The information you need to include is the type of physical activity, the time you start and the duration of the exercise. Please also indicate the intensity and changes in intensity if relevant.

Please also indicate below how you would generally describe your physical activity at work / study and at leisure. Please tick the most appropriate for each:

	Inactive	Sedentary (In care, very little activity)	Lightly Active (Office work, sitting/ standing activities)	Moderately Active (Active students, light industry, golf, housework)	Very Active (Full time athletes, mine/steel workers, soldiers, most sports, gardening, dancing)	Exceptionally Active (lumberjacks, female construction workers, running, climbing, football, basketball)
Work/study						
Leisure						

Example Food Diary and Activity Log entry

Here is an example of the level of detail needed when recording your daily diary. Please fill in as much as you can of your own diary on the following pages.

Day 1 DATE: / /			
BEFORE BREAKFAST			
Time	Food/Drink	Description & Preparation	Amount
7am	Water	From tap	300ml
BREAKFAST			
Time	Food/Drink	Description & Preparation	Amount
8am	Toast with butter and jam	Brown bread - thin sliced - without the crust - toasted With 'flora light' butter and 'bonne maman' strawberry jam	2 slices of bread (30g each without crusts), butter thinly spread, teaspoon of jam on each slice
	Apple	Pink Lady	85 g
	Orange juice	'Tropicana' orange juice - not from concentrate - with 'bits'	200ml
MID MORNING			
Food	Food/Drink	Description & Preparation	Amount
10am	peanuts	Unsalted 'sainsbury's own'	Small bag (50g)
	coffee	Caffeinated - from café, with semi-skimmed milk, white sugar	Regular size (300ml) small serving of milk (quite strong coffee), 1 teaspoon of sugar
LUNCH			
Time	Food/Drink	Description & Preparation	Amount
1.30pm	Ham and cheese sandwich	White thick sliced bread with 'flora light' butter, sainsbury's 'taste the difference' smoked ham, mild cheddar cheese, grated	2 slices of bread (30g each) butter thinly spread, 2 slices of ham (10g each), 20g cheese
	Chocolate chip cookie	From starbucks	Small cookie (55g)
MID AFTERNOON			
Time	Food/Drink	Description & Preparation	Amount
4.10pm	coffee	De-caffeinated - from café with semi-skimmed milk and white sugar	Regular size (300ml) small serving of milk (strong coffee), 1 teaspoon sugar
	Blueberry muffin	From 'tesco bakery'	Large muffin (178g)

EVENING MEAL			
Time	Food/Drink	Description & Preparation	Amount
6.30pm	Salmon	Grilled with olive oil, salt and pepper	1 medium sized fillet (250g) 1 tablespoon of oil pinch of salt and pepper
	Side salad	Iceberg lettuce, sliced tomatoes, sliced cucumber.	Handful of lettuce leaves, 2 medium sized tomatoes (25g each) half a cucumber (30g)
	Cream and mushroom sauce	double cream - 'tesco's own' 'sainsbury's own' closed top mushrooms - sliced	100ml double cream 150g mushrooms only had half the sauce
	Wine	'sainsbury's house dry white' wine	2 medium glasses (175ml each)
	Ice-cream	'ben and jerry's chocolate chip cookie dough'	3 scoops (about 1/3 of the 500ml tub)
LATER EVENING			
Time	Food/Drink	Description & Preparation	Amount
		n/a	
Other snacks and drinks (if not already recorded)			
Time	Food/Drink	Description & Preparation	Amount
Through h -out day	coke	'diet coke'	1 can (330ml)
	water	From tap - bottled	1 L

Exercise completed				
Time	Duration	Activity	Intensity	Notes/Comments
7pm	45 minutes	swimming	Slow pace - medium intensity	30 laps of 50 m

Other notes: eg. mood / sleep / concentration / fatigue

Day 1 DATE: / /			
BEFORE BREAKFAST			
Time	Food/Drink	Description & Preparation	Amount
BREAKFAST			
Time	Food/Drink	Description & Preparation	Amount
MID MORNING			
Food	Food/Drink	Description & Preparation	Amount
LUNCH			
Time	Food/Drink	Description & Preparation	Amount
MID AFTERNOON			
Time	Food/Drink	Description & Preparation	Amount

EVENING MEAL			
Time	Food/Drink	Description & Preparation	Amount
LATER EVENING			
Time	Food/Drink	Description & Preparation	Amount
Other snacks and drinks (if not already recorded)			
Time	Food/Drink	Description & Preparation	Amount

Exercise completed				
Time	Duration	Activity	Intensity	Notes/Comments

Other notes: eg. mood / sleep / concentration / fatigue

APPENDIX F: Whey protein isolate – product information

APPENDIX F1: Chapters 4a and 4b

Material	Whey Protein Isolate
Name	PSNU27600
Solids (%)	95.26
Protein (%)	82.93
Carbohydrates (%)	9.264
Ash (%)	2.72
Fat (%)	0.15

Amino acids

*	Trp. (g/100 g protein)	2.64
	Asp (g/100 g protein)	12.64
*	Thr(g/100g protein)	5.32
	Ser(g/100g protein)	4.63
	Glu(g/100g protein)	18.25
	Pro(g/100g protein)	5.33
	Gly(g/100g protein)	1.98
	Ala(g/100g protein)	5.14
*	Val(g/100g protein)	5.78
*	Isoleu(g/100g protein)	6.21
*	Leu(g/100g protein)	13.40
	Tyr(g/100g protein)	3.85
*	Phe(g/100g protein)	3.94
*	His(g/100g protein)	2.20
*	Lys(g/100g protein)	11.11
	Arg(g/100g protein)	2.74
	Cys + Cystine(g/100g protein)	3.20
*	Methionin(g/100 g protein)	2.43

**Essential amino acids*



Statement on status of PSNU 27600

Supplier: Arla Foods Ingredients Group P/S
Product: PSNU 27600
Revision no.: 1
Date of Issue: 8. maj 2013

To whom it may concern

PSNU 27600, produced by Arla Foods Ingredients Group, comply with the definition of a food, as stated in article 2 of Regulation (EC) No 178/2002 of 28 January 2002 regarding the general principles and requirements of food law: Food (or foodstuff) means any substance or product, whether processed, partially processed or unprocessed, intended to be, or reasonably expected to be ingested by humans. The product is produced at a plant which is authorised for the production of food in accordance with Regulation (EC) No. 853/2004, and comply with the relevant microbiological criteria, as stated in Regulation (EC) 2073/2005 on microbiological criteria for food.

Best regards
Arla Foods Ingredients Group P/S

A handwritten signature in purple ink that reads "Lisa Jensen".

Lisa Jensen
Regulatory & Risk Manager

Arla Foods Ingredients Group P/S
Senderhøj 10-12
DK-8260 Viby J
Denmark
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fax: +4586281838
E-mail: ingredients@arlafoods.com
arlafoodsingredients.com

APPENDIX F2: Chapters 5 and 6

Lacprodan® SP-9225 Instant



Description

Lacprodan® SP-9225 Instant is a soya lecithin instantised whey protein isolate for protein fortification in ready-to-use dry mixes for sports foods. The instant powder has an increased wettability and dissolves instantly in cold and warm water.

Properties

- Instant over a wide pH-range
- Designed for solutions with a neutral pH
- Neutral taste
- Low fat and carbohydrate content

Chemical specifications

Protein (Nx6.38) as is		87 %
Protein (Nx6.38) d.m.		92 %
Lactose	max.	1.0 %
Fat	max.	2.0 %
Ash	max.	4.5 %
Moisture	max	6 %

Minerals

Sodium Na	level	0.5 %
Phosphorus P	level	0.2 %
Chloride Cl	level	0.1 %
Potassium K	level	1.0 %
Calcium Ca	level	0.1 %

Nutritional data

Calculated values for nutrition labelling per 100 g powder

Energy	1499 kJ/357 kcal
--------	------------------

Physical specifications

pH (10% solution)		6.5-7.0
Scorched particles		disc A
Bulk density (tapped)	level	0.3 g/cm ³
Solubility index	max.	0.1 ml
Wettability	level	< 30 sec.
Dispersibility	level	> 95 %
Colour of powder		white to cream
Colour in 10% solution		opaque
Flavour/odour		bland
Soya lecithin added		

Microbiological specifications

Total plate count	max.	10,000 CFU / g
Enterobacteriaceae	max.	10 CFU /g
Staphylococcus aureus coagulase +	absent	in 1g
Yeast/Mould	max.	10 CFU /g
Salmonella	absent in	125 g

Packaging

Paper bags with a polyethylene inner liner containing 15 kg net.

Storage

Store in closed bags under cool and dry conditions to prevent deterioration due to humidity and high temperatures.

Shelf Life

Minimum 24 months if kept under the prescribed storage conditions.

Amino acids (AA)

Typical amino acid composition g AA/100 g protein

Alanine		5.6
Arginine		2.2
Aspartic acid (asparagine)		11.5
Cysteine (Cystin)		2.5
Glutamic acid (glutamine)		18.6
Glycine		1.7
Histidine	*	1.8
Isoleucine	*	7.1
Leucine	*	11.5
Lysine	*	10.3
Methionine	*	2.5
Phenylalanine	*	3.2
Proline		6.9
Serine		5.2
Threonine	∞	7.7
Tryptophene	∞	1.8
Tyrosine	∞	3.1
Valine	*	6.3
Total BCAA/TAA		22.7
* Essential Aminoacids		

Legal references

Lacprodan® SP-9225 Instant. The product is manufactured, packaged and labelled according to the relevant EU-regulations for food and food ingredients, and/or FAO/WHO Codex Alimentarius, when relevant. This includes that the milk/milk constituents used as raw material origins from healthy cows. The milk used in the production is included in monitoring programmes for undesirable substances, as required by regulations or HACCP-based risk assessment. The production plants are approved by the competent authorities and included in the EU-register of approved food establishments.

For products manufactured outside EU the products comply with relevant regulations in the country where the product is produced.



Arla Foods Ingredients Group P/S

Sanderhøj 10-12, 8260 Viby J, Denmark, Tel. +45 89 38 10 00, www.arlafoodsingredients.com

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APPENDIX G: Visual analogue scales (VAS)

Participant ID:

Visit:

Date:

Timepoint 1: Baseline

How hungry do you feel?

I am not hungry at all. | _____ | I have never been more hungry.

How full do you feel?

Not at all full. | _____ | Totally full.

How satisfied do you feel?

I am completely empty. | _____ | I cannot eat another bite.

How much do you think you can eat?

Nothing at all. | _____ | A lot.

APPENDIX H: Rating of perceived exertion (RPE) scale

6	No exertion at all
7	
8	Extremely light
9	Very light
10	
11	Light
12	
13	Somewhat hard
14	
15	Hard (heavy)
16	
17	Very hard
18	
19	Extremely hard
20	Maximal exertion

Borg-RPE-Scale®
© Gunnar Borg 1970, 1985, 1998



Instructions to the Borg-RPE-Scale®

During the work we want you to rate your perception of exertion, i.e. how heavy and strenuous the exercise feels to you and how tired you are. The perception of exertion is mainly felt as strain and fatigue in your muscles and as breathlessness or aches in the chest.

Use this scale from 6 to 20, where 6 means "No exertion at all" and 20 means "Maximal exertion."

- 9 Very light. As for a healthy person taking a short walk at his or her own pace.
- 13 Somewhat hard. It still feels OK to continue.
- 15 It is hard and tiring, but continuing is not terribly difficult.
- 17 Very hard. It is very strenuous. You can still go on, but you really have to push yourself and you are very tired.
- 19 An extremely strenuous level. For most people this is the most strenuous exercise they have ever experienced.

Try to appraise your feeling of exertion and fatigue as spontaneously and as honestly as possible, without thinking about what the actual physical load is. Try not to underestimate, nor to overestimate. It is your own feeling of effort and exertion that is important, not how it compares to other people's. Look at the scale and the expressions and then give a number. You can equally well use even as odd numbers.

Any questions?